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Metabolomic Fingerprinting of Food Plants by Nuclear Magnetic Resonance Spectroscopy and Gas Chromatography/Mass Spectrometry

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When we administer medicine, we administer the whole world: that is, all the virtue of heaven and earth, air and water. Therefore, your medicine must contain the whole firmament of both upper and lower spheres. Think with what energy the Nature takes heaven and earth with all their powers when she strives against death.

Theophrastus Bombastus von Hohenheim, 1530: *Das Buch Paragranum*

1. Introduction

1.1 '-Omics' technologies

Over the past few years, the '-omics' fields have seen an explosive growth opening new perspectives for biological research purpose. The development of analytical instrumentations, data processing and chemometric tools simplify the study of complex biological systems on a large-scale. Metabolomic, together with other '-omics' disciplines such as genomic, transcriptomic, and proteomic, is becoming an integral part of a system biological approach for investigating organisms. Fig. 1.1 reports the classification of the '-omics' technologies and the correlation among them. Although transcriptome represents the process for protein synthesis, an increase in mRNA levels does not always correspond with an increase in proteins due to numerous post-transcriptional regulation mechanisms (Kendrick 2014; Vogel et al. 2012). Therefore, changes in transcriptome or proteome do not always reflect alterations in biochemical phenotypes. For this reason, the association of metabolomic with the other analytical areas of genomic, transcriptomic and proteomic constitute a very powerful method to study biological systems.

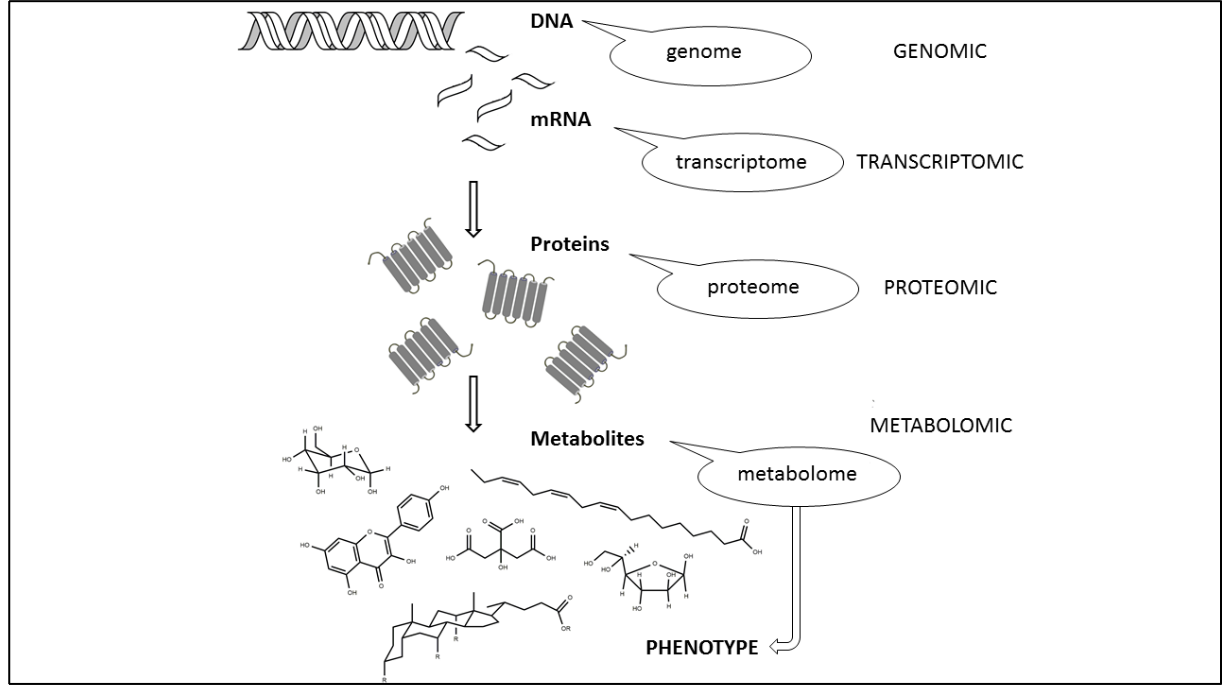


Fig. 1.1 General classification of '-omics' technologies

1.1.1 Genomic and transcriptomic

Genomic is considered a discipline of genetics able to define the overall genome of organisms, that includes genes, noncoding DNA and also the genomes from mitochondria and chloroplasts. On the other side, transcriptomic aims to determine the expression level of genes (mRNA and noncoding mRNA). Genome and transcriptome consist of linear polymers of five nucleotides with highly similar chemical properties. Several technologies have been developed to determine and quantify simultaneously a high number of genes and significant changes of their mRNA. In the late 90's and 2000's, DNA array technology progressed rapidly as a new method in which the hybridization plays an important role to measure the expression levels of genes (Bumgarner 2013). More recently, sequence-based approaches directly determine the cDNA sequences and the changing of the cellular transcriptome. Although RNA-seq is a tool still under development, it offers several advantages, such as the use of low amount of RNA, the ability to distinguish

allelic expressions and different isoforms and the high levels of reproducibility for both technical and biological replicates (Hrdlickova et al. 2017).

1.1.2 Proteomic

The number of proteins is normally higher than the number of genes and this happens because a single gene codes for multiple proteins (Vogel et al. 2012). For this reason, the proteome cannot be studied sufficiently by gene expression thus leading to the development of proteomic. This area of study aims to characterize and quantify which proteins are present in a biological organism under given conditions. One of the most used technique to analyse the proteome is the two-dimensional polyacrylamide gel electrophoresis (2-DE) that can differentiate a large number of proteins on the base of their relative mass and isoelectric point. Nowadays, the development of functional tools allows parallel analysis between gene and protein expression.

1.1.3 Metabolomic

Metabolomic is the '-omic' that study the whole metabolome in a cell, tissue or organism from both qualitative and quantitative point of view. The interest in using metabolomic for nutrition, agriculture, food science, human health and drug discovery has seen an exponential increase reaching a peak on 2016. Fig. 1.2 shows how the number of publications containing the term "metabolomic", taken from PubMed, is constantly growing.

In the metabolome, there are two kind of compounds, the primary metabolites and the secondary metabolites. The primary metabolites are compounds involved in the basic functions such as respiration, growth and maintenance of the cell. Basically, all organisms share the same type of primary metabolites. The secondary metabolites are species specific and play a role in the interaction of a cell with other cells or with environment. Secondary metabolites are responsible of plant flavour or colour and are associate to plant resistance against pests and diseases.

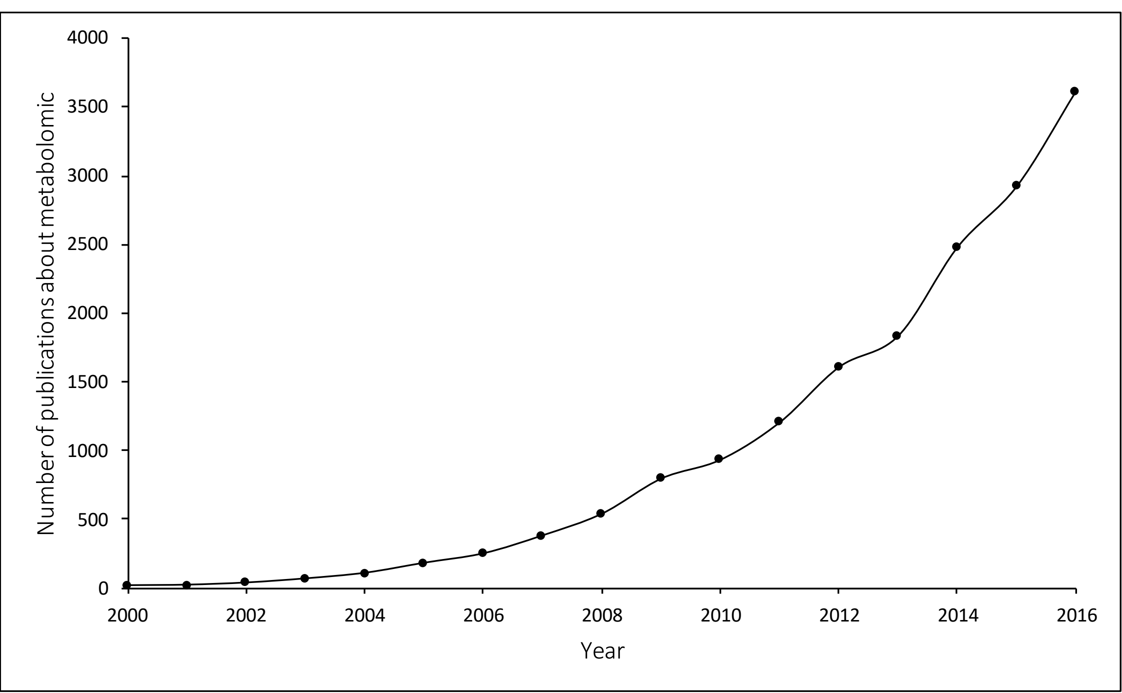


Fig. 1.2 A research on PubMed showing the number of publications containing the term "metabolomic"

The chemical complexity of metabolites ranges from ionic inorganic species to complex natural products, from hydrophilic to lipophilic compounds, from volatile to non-volatile molecules and from low to high molecular weight. Their production is not only regulated by gene expression but also by environmental conditions where metabolomic is able to define the biochemical phenotype of a cell or tissue.

During the metabolomic analysis, both primary and secondary metabolites will be detected after a snapshot of all metabolome and relative or absolute quantification can be done. This approach is applied on several topics which include:

- Studying fingerprints of different species, varieties, genotypes or ecotypes to obtain more information about taxonomy or biochemistry (Brahmi et al. 2015).
- Comparing several classes of metabolites in response to external chemical or physical treatments (Catola et al. 2016).
- Highlighting differences and similarities between the metabolite content of mutants or transgenic plants and that of their wild-type counterparts (Kristensen et al. 2005).

- Monitoring developmental processes such as metabolic transition from immature to ripe fruit (Aharoni et al. 2002).

Generally, in the metabolomic studies there are four critical steps (Fig. 1.3). The first one is sample preparation. At this step, it is necessary to control time and temperature which influence the accuracy and the reproducibility of results. Metabolic reactions are extremely rapid, so the freezing and storage of the sample at -80°C is required.

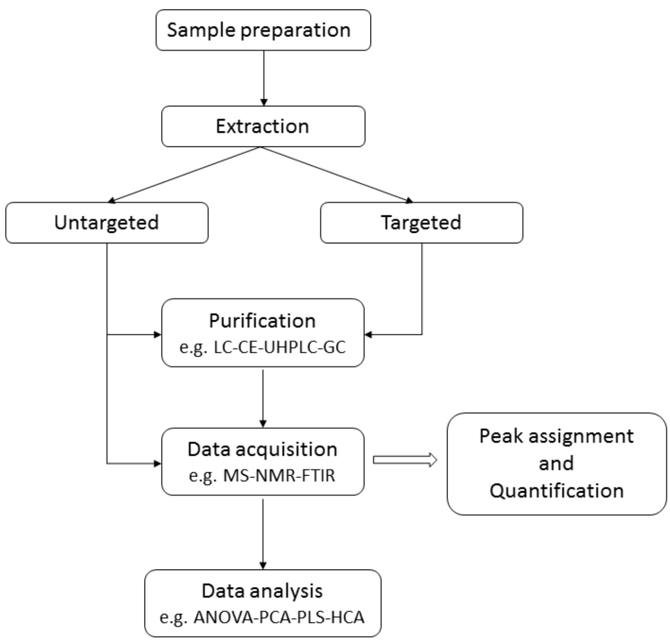


Fig. 1.3 Simplified workflow of the process for metabolomics analyses

The second step is the extraction that aims to maximize the number and the content of metabolites of interest. A recent study on *Arabidopsis thaliana* showed that a particular combination of MeOH/H_2O/$CHCl_3$ is the best solvents mixture in comparison to others (Gullberg et al. 2004). Ultrasonic treatment is also used as a disruption method to reduce the time and to improve the efficiency of the extraction. Following this, several detection techniques can be used to analyse the extracts. A high amount of data is generated from the acquisition and compounds identification; therefore, data analysis is the fourth crucial step, briefly discuss in paragraph 1.3. A further separation or purification of metabolites after their

extraction is also possible, but in many applications, samples are analysed directly. Whether this intermediate step is necessary, depends on the kind of research that is done. Nowadays, two main strategies are used for metabolomics investigations: untargeted (non-targeted) metabolomics and targeted metabolomics. The untargeted approach aims to compare the whole metabolite profiles among different sample groups, whereas targeted metabolomics focuses on a tiny fraction of the metabolome analysing a specific group of compounds related to a specific metabolic pathway (e.g., fatty acids, amino acids or phytochemicals) (Son et al. 2008; Vrhovsek et al. 2012). For this purpose, one or few metabolites are selected after a high level of purification and all other compounds are ignored.

1.2 Analytical approaches

Metabolomic aims to identify and quantify the overall metabolome. To achieve this objective, several analytical approaches can be used, each with their own advantages and disadvantages. They can be grouped as follows:

- chromatographic methods: liquid chromatography (LC), high-performance LC (HPLC); ultra-high performance LC (UHPLC), gas chromatography (GC), capillary electrophoresis (CE), thin layer chromatography (TLC);
- mass spectrometry (MS);
- spectroscopy: nuclear magnetic resonance spectroscopy (NMR); Fourier transform infra-red (FT-IR); ultraviolet methods (UV).

Each technique, mentioned above, provides two steps. The first step is a qualitative study in which the signals observed are assigned to a specific metabolite with the help of standard compounds, data literature and different libraries (Smith et al. 2005; http://www.hdscience.com). Consequently, the second step is a measurement of relative or absolute amounts of each single component based on calibration curves of internal standard. A

compromise between speed, selectivity and sensitivity should be found between all these methods to select the most suitable approach, although a combination of two or more of them is also used to provide complementary information and to reduce sample complexity. For example, LC/MS with atmospheric pressure ionization (API) is also applied to metabolomic study, but only a relatively small number of analytes can be detected. Specifically, the production of pseudo-molecular ions ($[M+H]^+$ or $[M-H]^-$) depends on several factors and it is not always easy to predict which one will be produced. In addition to this, many compounds do not ionize optimally and for this reason LC/MS is more suited for metabolites which ionize similarly under the same condition. A rapid and non-destructive technique is FT-IR based on the stretching and bending vibration of chemical bonds irradiated by the light (usually 4000–400 cm^{-1}). Although it does not require a difficult preparation of the sample, its sensitivity is not as high as that of the other methods. However, in the last few years, several studies have been undertaken using FT-IR to diagnose disease or dysfunction and to acquire metabolic profiling of body fluids (Lemes et al. 2016; Isogawa et al. 2014). On the contrary, in other research fields, such as food chemistry, metabolomics studies with NMR and MS have been mostly applied. For this reason, the limitations and advantages of ^1H NMR and GC-MS, which have been used in this study to analyse the whole metabolome of two plants extracts, will be discussed below. In detail, NMR spectroscopy is a physical measurement of the resonances of atoms with a non-zero magnetic moment, such as ^1H, ^{13}C, ^{15}N, ^{19}F, ^{31}P in a strong magnetic field. The application of a magnetic field on the nuclei of these atoms causes the promotion of electrons from low-energy to high-energy spin states and the subsequent emission of radiation during the relaxation process. It is a non-destructive technique with high reproducibility in which compounds and extracts have a highly specific spectrum. Its sensitivity can be improved by the application of high and uniform magnetic fields (frequencies commonly used range between 300-700 MHz) and also by the use of a cryogenic probe-heads. The sensitivity of NMR also depends on the abundance of isotope studied and time of analysis. The most sensitive

isotopes are ^{1}H, ^{19}F and ^{31}P, but the last two nuclei can only be found in a restricted number of compounds. For this reason, ^{1}H is the preferred one to study metabolites fingerprinting. The only variables are the solvents used which depend on the polarity of the extract that should be analysed. In the ^{1}H NMR sample there is always much more solvent than substance, so deuterated solvent, such as D_2O, CD_3OD, $CDCl_3$, should be used to minimize the signal solvent in the spectra. The chemical shifts in ^{1}H NMR spectra (0-10 ppm) are assigned to a specific metabolite. The signals can be reported along two frequency axis to produce a two-dimensional spectrum (2D) which can be either homonuclear, if the correlation comes from the same nucleus (usually ^{1}H-^{1}H), or heteronuclear if on the frequency axis there are 2 different nuclei (usually ^{1}H-^{13}C).

A more sensitive tool is GC-MS, which is widely applied in metabolomic because of its high specificity and sensitivity for suitable analyte classes. This technique, similarly to the LC/MS, is based on the separation and detection of ions according to their mass-charge (m/z) ratio. A limitation of gas chromatography coupled with mass spectrometry is that not all injected components will pass through the column, because of different physico-chemical properties of the analytes (polarity, stability, molecular mass, volatility, etc). Components can therefore remain in the injector or in the column, causing the whole system to respond differently after each injection. However, this can be avoided if all samples are derivatized at room or elevated temperature before the analysis in order to reduce their polarity. After derivatization, the volatility and thermal stability of the compounds is also provided allowing their elution at high temperatures without decomposition.

In particular, compounds containing active hydrogens, such as -OH, -SH, -NH and -COOH should be derivatized prior to GC-MS analysis. Derivatization can be achieved by three general reactions, which are alkylation, acylation and silylation (Orata et al. 2012). Usually, silylation is the most suitable reaction for non-volatile compounds for GC analysis. As shown in Fig. 1.4 (A and B), after silylation, an active hydrogen is substituted by a silyl group. Prior to silylation,

an oximation reaction is recommended in polar extracts, especially if there could be sugars in the sample. It is well known that sugars like fructose and glucose have different structures in a water solution with a predominance of the cyclic form. To reduce the tautomerism, which can produce multiple peaks for the same compound on the chromatogram, the oximation is required (Shepherd et al. 2007) and the carbonyl functional groups of aldehydes and ketones will be converted in oximes (see the reaction in Fig. 1.4 C and D). There are several oximation and silylation reagents which can be used, but recent studies have shown that methoxyamine hydrochloride and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide are the most appropriate for metabolomics studies (Dettmer et al. 2007; Ruiz-Matute et al. 2011).

These reactions are moisture sensitive, for this reason the sample must be completely dried. Another advantage of using GC-MS is that a very small amount of derivatised samples is analysed (typical injection volumes of 1 µl) to give high resolution spectra.

In conclusion, GC-mass spectrometry and NMR spectroscopy have limitations and advantages. GC-MS has a detection threshold of 10^{-12}mol, which is more sensitive of ^{1}H NMR spectroscopy where a value of 10^{-6}mol is observed (Sumner et al. 2003). In both analytical methods, extraction step is required, but all classes of compounds need to be derivatized in GC-MS analysis, which takes additional time, processing, and variance in comparison to the NMR tool.

Fig. 1.4 Derivatization steps prior to GC-MS analysis: (A) silylation reactions between hydrophilic groups and trimethylsilyl (TMS) reagent (Nu = nucleophile). (B) silylation reaction mechanism. (C) oximation reaction of aldehyde and ketone (e.g. open chain form of glucose and fructose). (D) reaction mechanism between methoxyamine hydrochloride and a generic aldehyde.

1.3 Data analysis

The untargeted metabolomic data, obtained by the identification and quantification of as many metabolites as possible, is subsequently statistically processed. Statistical approaches require replicates of samples preparation. Usually, multivariate data analysis (MVDA) techniques are used to maximize classification of samples. Principal components analysis (PCA), partial least square (PLS), hierarchical cluster analysis (HCA) and self-organizing mapping (SOM) have been extensively employed as statistical tools for metabolomics. PCA is a useful approach to reduce the dimensionality of a large data set and is the most used chemometric tool as clustering technique. PCA describes the variance between original variables (in this case metabolites

concentrations) through a linear combination of new variables, which are principal components (PC), identifying how samples are different from each other and which variables (metabolites concentrations) contribute most to this difference. On the contrary, PLS regression is more used to create a prediction model in metabolomics studies (Tarachiwin et al. 2008). HCA is also used frequently in metabolomics and measures the distance between rows or columns of a data matrix. This approach can be employed with an agglomerative or a divisive method and data are presented in a diagram known as dendrogram. SOM is a noncluster method widely used for genomic and transcriptomic (Abe et al. 2003; Hirai et al. 2004), but more recently it was also applied to monitor metabolic dynamics in rice leaves (Sato et al. 2008).

1.4 Aim of the study

Metabolomic analysis of food plants allows to obtain a fingerprinting of plant extracts by using different techniques, such as NMR spectroscopy and mass spectrometry, in order to obtain a snapshot of the metabolome of different groups of samples. This dissertation gave rise to three objectives.

The first objective was to characterise the metabolic profile of fourteen artichoke populations belonging to "Romaneschi" group (*Cynara carduncululus* L. var. *scolymus* L. Fiori) and one cultivated cardoon (*Cynara cardunculus* L. var. *altilis* DC) all collected in the Campania region of Italy. The investigation of the major nutraceuticals in artichoke using ^{1}H NMR was recently applied to Jerusalem artichoke (*Helianthus tuberosus* L.) (Clausen et al. 2012) but to the best of our knowledge this is the first time that proton NMR spectroscopy is used to provide a metabolic fingerprinting of *C. cardunculus*.

The second objective was to evaluate and compare the chemical composition of wild-types and mutant samples of chia seeds (*Salvia hispanica* L.). While, chia is a short-day flowering specie and it can produce seeds only in a restricted range of latitudes, breeding efforts have produce longer-day genotypes in order to extend this crop to other areas (Jamboonsri et al. 2012).

Mutations are introduced to wild-type chia seeds at University of Kentucky Research Foundation (Lexington, US) and more detailed information is made available under patent application number US 20130007909 A1. Mutant genotypes were made available from University of Kentucky to University of Naples Federico II through an agreement with University of Basilicata. A comparative analysis between commercial short-day flowering genotypes and mutant chia seeds was carried out in order to define possible differences in the chemical composition due to mutations. The analysis was also extended to two samples of chia seeds wild-types grown in Basilicata (Southern Italy) in order to evaluate the effect of fertilization with mineral nitrogen, on the metabolite composition.

The third objective is to determine the biological effect of irrigation on the metabolome of two genotypes of chia seeds. For this purpose, the chemical composition and antioxidant activity of a short-day flowering commercial chia genotype and a long-day flowering mutant grown with additional irrigation were compared to those of the untreated samples. This last experimental plan was developed during a visiting period of 5 months at the Abertay University of Dundee (Scotland, UK).

The study is organised in seven Chapters, where Chapters 2-6 are written as self-contained research papers.

References

Abe, T., Kanaya, S., Kinouchi, M., Ichiba, Y., Kozuki, T., & Ikemura, T. (2003). Informatics for unveiling hidden genome signatures. Genome research 13, 693-702.

Aharoni, A., Ric de Vos, C.H., Verhoeven, H.A., Maliepaard, C.A., Kruppa, G., Bino, R., Goodenowe, D. (2002). Non-targeted metabolomic profiling using Fourier transform ion cyclotron mass spectrometry (FTMS). OMICS: A Journal of Integrative Biology 6, 217–234.

Brahmi, N., Scognamiglio, M., Pacifico, S., Mekhoukhe, A., Madani, K., Fiorentino, A., & Monaco, P. (2015). ^{1}H NMR based metabolic profiling of eleven Algerian aromatic plants and evaluation of their antioxidant and cytotoxic properties. Food Research International 76, 334-341.

Bumgarner, R. (2013). Overview of DNA microarrays: types, applications, and their future. Current protocols in molecular biology 22-1.

Catola, S., Marino, G., Emiliani, G., Huseynova, T., Musayev, M., Akparov, Z., & Maserti, B. E. (2016). Physiological and metabolomic analysis of *Punica granatum* (L.) under drought stress. Planta 243, 441-449.

Clausen, M.R., Bach, V., Edelenbos, M., & Bertram, H. C. (2012). Metabolomics reveals drastic compositional changes during overwintering of Jerusalem artichoke (*Helianthus tuberosus* L.) tubers. Journal of Agricultural and Food Chemistry 60, 9495-9501.

Dettmer, K., Aronov, P.A., & Hammock, B.D. (2007). Mass spectrometry-based metabolomics. Mass spectrometry reviews 26, 51-78.

Gullberg, J., Jonsson, P., Nordstrom, A., Sjostrom, M., & Moritz, T. (2004). Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/ mass spectrometry. Analytical Biochemistry 331, 283-295.

Hirai, M.Y., Yano, M., Goodenowe, D.B., Kanaya, S., Kimura, T., Awazuhara, M., Arita, M., Fujiwara, T., & Saito, K. (2004). Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proceedings of the National academy of Sciences of the United States of America 101, 10205-10210.

Hrdlickova, R., Toloue, M., & Tian, B. (2017). RNA-Seq methods for transcriptome analysis. Wiley Interdisciplinary Reviews: RNA, 8(1).

Isogawa, M., Yoshida, S., & Shimozawa, N. (2014). Evaluation of Fourier Transform Infrared Spectroscopy for Diagnosis of Peroxisomal Diseases with Abnormal Very-Long-Chain Fatty Acid Metabolism. American Journal of Analytical Chemistry 5, 359.

Jamboonsri, W., Phillips, T.D., Geneve, R.L., Cahill, J.P., & Hildebrand, D.F. (2012). Extending the range of an ancient crop, *Salvia hispanica* L. – a new ω3 source. Genetic Resources and Crop Evolution 59, 171-178.

Kendrick, N. (2014). A Gene's mRNA Level Does Not Usually Predict its Protein Level. Madison, WI: Kendrick Laboratories.

Kristensen, C., Morant, M., Olsen, C. E., Ekstrøm, C. T., Galbraith, D. W., Møller, B. L., & Bak, S. (2005). Metabolic engineering of dhurrin in transgenic Arabidopsis plants with marginal inadvertent effects on the metabolome and transcriptome. Proceedings of the National Academy of Sciences of the United States of America 102, 1779-1784.

Lemes, L. C., Júnior, C., Cesar, P., Strixino, J. F., Aguiar, J., & Raniero, L. (2016). Analysis of serum cortisol levels by Fourier Transform Infrared Spectroscopy for diagnosis of stress in athletes. Research on Biomedical Engineering 32, 293-300.

Orata, F. (2012). Derivatization reactions and reagents for gas chromatography analysis advanced gas chromatography. In: Advanced Gas Chromatography - Progress in Agricultural, Biomedical and Industrial Applications. (Ed. M.A. Mohd) ISBN: 978-953-51-0298-4 In Tech Europe

Ruiz-Matute, A. I., Hernandez-Hernandez, O., Rodríguez-Sánchez, S., Sanz, M. L., & Martínez-Castro, I. (2011). Derivatization of carbohydrates for GC and GC–MS analyses. Journal of Chromatography B 879, 1226-1240.

Sato, S., Arita, M., Soga, T., Nishioka, T., & Tomita, M. (2008). Time-resolved metabolomics reveals metabolic modulation in rice foliage. BMC systems biology 2, 51.

Shepherd, T., Dobson, G., Verrall, S.R., Conner, S., Griffiths, D.W., McNicol, J.W., Davies, H.V., & Stewart, D. (2007). Potato metabolomics by GC–MS: what are the limiting factors? Metabolomics 3, 475-488.

Smith, C.A., O'Maille, G., Want, E.J., Qin, C., Trauger, S.A., Brandon, T.R., Custodio, D.E., Abagyan, R., & Siuzdak, G. (2005). METLIN: a metabolite mass spectral database. Therapeutic drug monitoring 27, 747-751.

Son, H. S., Kim, K. M., Van Den Berg, F., Hwang, G. S., Park, W. M., Lee, C. H., & Hong, Y. S. (2008). ^{1}H nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. Journal of Agricultural and Food Chemistry 56, 8007-8016.

Sumner, L. W., Mendes, P., & Dixon, R. A. (2003). Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62, 817-836.

Tarachiwin, L., Masako, O., & Fukusaki, E. (2008). Quality evaluation and prediction of *Citrullus lanatus* by ^{1}H NMR-based metabolomics and multivariate analysis. Journal of Agricultural and Food Chemistry 56, 5827e5835.

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics 13, 227-232.

Vrhovsek, U., Masuero, D., Gasperotti, M., Franceschi, P., Caputi, L., Viola, R., & Mattivi, F. (2012). A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. Journal of Agricultural and Food Chemistry 60, 8831-8840.

2. Artichoke: botanical, agronomical, phytochemical and pharmacological overview*

2.1 Introduction

The species *Cynara cardunculus* is a component of the Mediterranean diet and consists of the globe artichoke, *C. cardunculus* subsp. *scolymus*, the cultivated cardoon, *C. cardunculus* subsp. *altilis*, and the wild cardoon, *C. cardunculus* subsp. *sylvestris*. The globe artichoke, formerly named *Cynara scolymus* L. is a perennial herbaceous crop, originating from the Mediterranean region, where it has been grown for thousands of years and from where it was diffused all over the world. The scientific name of the plant originates from the Greek ‘‘skolymos’’ meaning pointed stake, because of its spines, whereas ‘‘Kynara’’ possibly comes from the name of an Aegean Island where it was grown or from the recommendation of Columella to fertilize this crop with ashes (Cineres) (Chevallier 1996). The common name of artichoke comes from the Arabic ‘‘al Quarshuff’’. Artichoke was used as food and medicine by ancient Egyptians, Greeks, and Romans. In the fourth century AD the greek Teophrastus reports that artichoke was grown in Sicily and in thereafter the Egyptian king Ptolemy Euergetes recommended his army to eat artichokes because they were considered a source of strength and braveness. Historical sources include mosaics from the roman empire at the Bardo Museum of Tunis, and the roman authors Varo, Plinius, Columella and Dioscorides, who recommended the application of mashed roots on the body to sweeten offensive odours (Chevallier 1996). Between 800 and 1500 AD several sources report that artichokes brought from North Africa were grown in Sicily and Spain, and as a rarity in Florence and Venice, and in the same period breeding was

* de Falco, B., Incerti, G., Amato, M., & Lanzotti, V. (2015). Artichoke: botanical, agronomical, phytochemical, and pharmacological overview. *Phytochemistry reviews*, *14*(6), 993-1018.

conducted in monasteries in Europe. In his ''Italian Journey'' Goethe reports that Italian farmers eat artichokes as a curiosity, but artichoke was known in Europe since the fifteenth century and in the eighteenth and nineteenth century it was brought to the Americas by European immigrants. Production of artichokes amounts to 1,793,015 tones year^{-1} (FAO 2013), more than 60% of which in Europe (FAO 2013). The first world producer is Italy, and the country where artichoke production has most recently acquired commercial importance is China. The edible parts of the plant are large immature inflorescences, named capitula or heads, with edible fleshy leaves (bracts) and receptacle that has been shown to be a rich source of bioactive compounds (Fratianni et al. 2007; Lattanzio et al. 2009) and used as herbal medicine since ancient times for their beneficial and therapeutic effects. Artichoke has been used in the folk medicine against several diseases, such as hepatic diseases, jaundice, dyspepsia, chronic albuminuria, postoperative anemia, and used as diuretic and liver tonic (Schauenberg and Paris 1977). Extracts from artichoke have been used for hepatoprotection (Adzet et al. 1987) as a choleretic (Preziosi et al. 1959) and lipid-lowering agents (Gebhardt 1998). The flower head, cooked and eaten as a delicacy, contains a sweetener taste enhancing flavour perception, while the leaves contain a bitter taste used in the preparation of aperitif liqueurs (Fleming 1998). Nutritional and pharmacological properties of artichoke heads and leaves are attributed mainly to caffeoylquinic acid compounds and inulin present at high concentration. Other classes of chemical compounds, including flavonoids, anthocyanins, sesterpenes, triterpenes have been also found in the plant at lower amounts. In this paper the chemical composition of the artichoke has been reviewed with particular attention to the agronomical and pharmacological importance of the plant and to the methods of analysis, including the recently developed metabolomic studies.

2.2 Origin and classification

Included in the *Astereaceae* or *Compositae* family, subfam. *Tubuliflorae*, tribe *Cynareae*, the genus *Cynara* is made of diploid (2n = 2x = 34) species. According to molecular data, a wild ancestor of all species in the *Cynara* genus moved from the Mediterranean coast to the region of Sahara during the 4th glaciations of Pleistocene (Pignone and Sonnante 2009), due to its high thermal requirements. Towards the end of the glaciations (between 30,000 and 20,000 years ago) it moved back to the north coast of Africa and around 18,000 years ago it started to differentiate, and two groups of species were originated. In both groups genotypes did not equally diffuse in the whole Mediterranean basin, therefore some species and subspecies are preferentially found in the west and others in the centre-east regions. According to Pignone and Sonnante (2009), the first group includes seven wild species:

- C. *baetica* (Spreng.), C. *algarbiensis* Coss. ex Mariz, C. *humilis* L. more widespread in the West Mediterranean regions;
- C. *syriaca* Boiss, C. *cornigera* Lind., C. *cyrenaica* Maire and Weiller, in the Center-East of the Mediterranean basin;
- C. *aurantica* Pos., more equally distributed than other species.

The second group encompasses the *C. cardunculus* complex of species:

- *C. cardunculus* (L.) subsp. or var. *scolymus* (L) Hegi, the globe artichoke
- *C. cardunculus* (L.) subsp. or var. *altilis* DC, the cultivated cardoon,
- *C. cardunculus* (L.) subsp. *sylvestris* Lam., the wild artichoke or cardoon. This subspecies has been further divided into two types with different genetic pools and geographical distribution: one located in the Western Mediterranean basin, which is likely to be the progenitor of cultivated cardoon due to a breeding pressure towards larger leaves; the other located in Central-East Mediterranean, which is probably the

ancestor of globe artichoke due to a breeding pressure towards a larger inflorescence (Pignone and Sonnante 2009).

Within the whole *C. cardunculus* complex, intercrossing is entirely possible since all genetic types share the same primary gene pool, and are therefore interfertile, whereas only the secondary gene pool is shared within the whole *Cynara* genus (Rottenberg and Zohary 1996). Domestication of globe artichoke can be dated back to around the Roman Imperial age possibly in Sicily, whereas cultivated cardoon was likely domesticated in Spain in the middle ages. Food uses of cultivated cardoon are related to the large stems and leaf petioles. Propagation is largely based on seeds contained in a dry indehiscent fruit typical of the *Asteraceae* family. The edible part of globe artichoke is the capitulum or head, an inflorescence which is eaten immature, before flowering. Propagation is traditionally vegetative. The richest cultivated primary gene-pool of globe artichoke is found in Italy, where domestication occurred (Pignone and Sonnante 2004). To date more than 120 varietal types are reported (Lanteri and Portis 2008), and classification is based on harvest time and capitulum morphological traits (Portis et al. 2005). Edible heads may be produced in autumn and spring by early varieties, and only in spring-summer by late varieties. Four variety groups are described based on capitulum morphology:

1. the Spinosi group, bearing spines on capitulum bracts and leaves;
2. the Violetti group, with purple and less spiny heads;
3. the Romaneschi group, with spherical or subspherical non-spiny heads;
4. the Catanesi, with small, elongated and non-spiny capitulum.

Another classification is found in France with two main groups (Breton with large green capitula and Midi with smaller pigmented capitula). Many local landraces are found, and both phenotypic and genetic diversity are large even within varietal groups and populations (Portis et al. 2005), and this has been ascribed to traditional methods of propagation, based on on-farm vegetative reproduction, resulting in multi-clonal populations (Portis et al. 2005), and on accidental crossing with local wild artichokes, which also exhibit a high genetic variability. The

absence of spines in cultivated types is due to a dominant allele, whereas yield traits have a polygenic determination, and are strongly affected by the environment and management techniques, especially fertilization and water availability. Capitula pigmentation is due to antocyanic compounds, determined by a few genes and affected by the onset of cold temperatures as well as fertilization. Other quality traits are linked to genetics (Fratianni et al. 2007), environmental and agronomical factors, and are affected by plant phenological stage.

2.3 Morphology, ecology and use

Artichoke is an allogamous and entomophylous erect herbaceous perennial, with lifecycle exceeding 10 years, which may be reduced to 2–4 years due to loss of productivity in intensive growing systems. The stem is very short and leaves may reach 50–200 cm of length. The inflorescence (capitulum or head) consist in a very long peduncle, (up to 180 cm), a receptacle where flowers are inserted, and external bracts. A main head and 4–20 secondary and tertiary heads are produced per plant. Heads are harvested in the early stages of their development and represent 30–40% of artichoke fresh weight. Considering that only the central portion is eaten, the ratio of edible fraction/total biomass decrease to 15–20% of total plant biomass. This ratio decreases further if the contribution to the total biomass represented by offshoots, removed from the field by common cultural procedures, is also considered (Marzi and Lattanzio 1981; Lattanzio 1982). Thus, leaves, external bracts and stems discarded by the artichoke processing industry, represent a huge amount of discarded material (about 80–85% of the total biomass of the plant), which could be used as a source of food additives and nutraceuticals (Llorach et al. 2002; Lopez-Molina et al. 2005) or as a raw material in the green chemistry industry such as paper–pulp production, biofuels or plant dyes (De Falco and di Novella 2011). This is commercially viable given the high biomass production of the plant, up to 33 t ha^{-1} (Archontoulis et al. 2010).

Flowers contain proteolytic enzymes and act as natural coagulants used as a substitute of rennet (Amato et al. 2011) and plant stems and leaves are traditionally a forage (Christaki et al. 2012). The dry indehiscent fruits (achenes) have a high content of polyphenols and other antioxidants so they have been proposed for industrial extraction (Durazzo et al. 2013).

The plant has a large root system which may reach up to 5 m (Archontoulis et al. 2010) and amounts to about 50% of the residual plant biomass after head harvest (Raccuia et al. 2004). After head harvest plant leaves are left on the plant to replenish root reserves with photosynthates and thereafter grazed or chopped and vegetation restarts from basal gems at the expenses of below-ground reserves after the first fall precipitations or summer irrigation. Due to its role of both resource uptake and reserve, the root contains a percentage of sugars of about 25%, 89.4% of which is inulin (Raccuia et al. 2004), an important prebiotic compound.

Offshoots from basal gems need to be thinned, and are traditionally used for vegetative propagation. Due to problems of genetic variability and infections, and the high labour requirements of vegetative reproduction, in vitro culture or seed propagation are increasingly used. Seed production occurs through free pollination (producing a high genetic, phenotypic and phenological variability) or creation of F1 hybrids which are more uniform in behaviour.

Artichoke phenology has been described by Archontoulis et al. (2010) according to the BBCH scale and Virdis et al. (2009, 2014) have studied the genetic factors and environmental requirements for timing of leaf emission (phyllochron) and head production. Flower induction requires a critical photoperiod of at least 10.5 h and a thermal sum of at least 200–250 °C with a base temperature of 7–9 °C, and artichoke does not survive at less than -10 °C (Bianco 1990). At high temperatures (>30 °C) the quality of edible heads decreases. The plant is moderately tolerant to salinity with genotypic variability, and may endure values of soil electrical conductivity between 2.6 and 6.1 dS m^{-1} without yield losses (Cantore and Boari 2009). Artichokes are grown in consociation with other Mediterranean crops such as olive orchards or in specialized fields (Fratianni et al. 2007). In both systems artichoke plants provide ecological

services like carbon storage and an effective form of land protection from erosion since they develop a considerable ground cover due to a large leaf area reaching a Leaf Area Index up to 6 m^2 of leaves per m^2 of soil, and a root density of over 10 cm of roots per cm^3 of soil (Amato et al. 2011).

2.4 Nutritional quality

From a nutritional point of view globe artichoke heads have around 7% of carbohydrates, 3% of proteins and less than 0.3% of lipids. Due to the high content of fiber (5.5%) and nutraceuticals (see following sections) artichoke is also a functional food and a folk medicine. Nutritional value of artichoke heads is due to its chemical composition characterized by high levels of phenolic compounds. Caffeoylquinic acids are the main phenolic compounds of artichoke. The other phenolics belong to the classes of flavonoids and anthocyanins (see following sections).

The 75% of the total sugar content in artichoke edible parts is attributed to the water-soluble polysaccharide inulin whose structure and biological activities are described in a following section. In addition, the content of inulin increase with the plant development reaching the 30% of the edible portion in artichoke heads of marketable quality (Lattanzio et al. 2009). Inulin has considered a dietary fiber because, reaching the colon as intact molecule, serve as substrate for bifidobacteria growth and makes them the predominant species (Robenfroid 1999).

2.5 Chemical constituent

Artichoke is a rich source of polyphenolic compounds, mainly caffeoylquinic acids and flavonoids, isolated in the polar extracts of the plant, together with the polysaccharide inulin. Concerning the lipophilic fraction, it is composed by fatty acids, triterpenes and sesquiterpenes as major metabolites. The amounts of such components are extremely variable and may depend on several aspects, such as environment, genetic factors, stress, harvest time, agronomical

processes, parts of artichoke analysed (inner, intermediate or outer bracts, receptacles, stems, capitula and leaves) as well as by different drying method (Lattanzio and Morone 1979; Lattanzio and Van Sumere 1987; Lombardo et al. 2010).

2.6 Caffeoylquinic acids

Table 2.1 summarizes the caffeic acids derivatives isolated from artichoke whose chemical structure is reported in Fig. 2.1. This class of compounds, named also hydroxycinnamic acids, is composed by a wide range of derivatives, of which chlorogenic and 1,5-O-dicaffeoylquinic acids are the most abundant components (Wang et al. 2003; Schütz et al. 2004; Pandino et al. 2013). The characterization of phenolic compounds between leaves, outer bracts, heads and stems was compared by Romani et al. (2006) on the two globe artichoke accessions, Violetto di Toscana and Terom. They reported caffeoylquinic acids both in leaves and stems with 1,5-dicaffeoylquinic acid as the most abundant compound. Higher amounts of chlorogenic acid was also found by Fratianni et al. (2007) in the inner bracts of three different genotypes of globe artichoke, Tondo di Paestum, Bianco di Pertosa and Violet de Provence. Analogous data have been reported by Pandino et al. (2011b) that found such compounds at higher amounts in the artichoke receptacles than in the outer bracts.

2.6.1 Biological activity of caffeoylquinic acids

Table 2.2 shows the biological activity of the isolated caffeoylquinic acids that are responsible of the beneficial effects demonstrated for artichoke. One of the most important activity of this class of compounds is ascribed to their antioxidant activity and Pandino et al. (2011a) demonstrated that this property is correlated with the content of caffeoylquinic acids which are the main components of the floral stem.

It is well known that caffeoylquinic acids have several biological effects and among them a higher inhibition of lipoxygenase has been found (Nishizawa and Fujimoto 1986; Nishizawa et al. 1987).

Gebhardt (1997) reported the activity of artichoke extracts as antioxidant and protective against hydroperoxide-induced oxidative stress with chlorogenic and 1,5 dicaffeoylquinic acids that were the main responsible of this effect. Fritsche et al. (2002) showed radical scavenging activities on DPPH (1,1-diphenyl-2-picrylhydrazyl) of chlorogenic acid and 3,5-dicaffeoylquinic acid. The anti-oxidative and anti-apoptotic activity of the latter molecule in HMEC-1 (human dermal microvascular endothelial cells) was reported by Zha et al. (2007), where the concentration of MDA decreases with administration of 3,5-dicaffeoyl quinic acid due to its scavenging of intracellular ROS. In addition, the level of apoptotic cells treated with this compound also decreases due to the inactivation of the enzyme caspase-3. More recently, the antioxidant activity of chlorogenic acid was also reported by Sato et al. (2011).

The antioxidant activity was also studied in human leukocytes by Pérez-Garcìa et al. (2000) who reported the inhibition of oxidative stress generated by reactive oxygen species (ROS) by cynarin, caffeic acid, chlorogenic acid and by the flavonoid luteolin.

Concerning 1,5-dicaffeoylquinic acid, Cao et al. (2010) demonstrated its protection of astrocytes from cell death in an in vitro model of ischemia/reperfusion. Recently, Xiao et al. (2011) indicated the dose-dependent neuro-protective mechanism of this compound against amyloid β 1–42 that induces apoptosis on neuronal culture.

Furthermore, cynarin, one of the most important caffeoylquinic acid, but not the most abundant one, reveals choleretic activity (Gebhardt 1997) and hepato-protective properties (Adzet et al. 1987) and it was demonstrated that it does not compromise transaminase activity.

The antibacterial activities of leaves extracts were also tested by Falleh et al. (2008), reporting activity against *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus*, and *Escherichia*

coli, and no activity against *Salmonella thyphymurium*. This antibacterial activity may be related to the high level of phenolic components in the leaves.

	R_1	R_3	R_4	R_5
Mono-caffeoylquinic acid				
Quinic acid	H	H	H	H
1-O-caffeoylquinic acid	X	H	H	H
3-O-caffeoylquinic acid	H	X	H	H
4-O-caffeoylquinic acid (cryptochlorogenic acid)	H	H	X	H
5-O-caffeoylquinic acid	H	H	H	X
Dicaffeoylquinic acid				
1,3-O-dicaffeoylquinic acid (cynarin)	X	X	H	H
1,4-O-dicaffeoylquinic acid	X	H	X	H
1,5-O-dicaffeoylquinic acid	X	H	H	X
3,4-O-dicaffeoylquinic acid	H	X	X	H
3,5-O-dicaffeoylquinic acid	H	X	H	X

Fig. 2.1 Chemical structures: caffeoylquinic acids

Table 2.1 Phenolic compounds isolated from artichoke

Compounds	Species	References
Caffeoylquinic acids		
1-O-caffeoylquinic acid	*Cynara scolymus* Green globe, Imperial Star, Violet	Wang et al. (2003)
	Cynara scolymus Violetto di Toscana and Terom	Romani et al. (2006)
Chlorogenic acid (common: 3-O-caffeoylquinic acid; IUPAC: 5-O-caffeoylquinic acid)	*Cynara scolymus* L.	Adzet and Puigmacia (1985)
	Cynara cardunculus scolymus Tondo di Paestum, Bianco di Pertosa, Violet de Provence, Violetto di Toscana.	Fratianni et al. (2007), Romani et al. (2006)
	Cynara cardunculus (L.) subsp. *scolymus* Hayek	Garbetta et al. (2014)
Cryptochlorogenic acid (4-O-caffeoylquinic acid)		
Neochlorogenic acid (5-O-caffeoylquinic acid)	*C.c altilis*, *C.c. scolymus* Blanc Hye`rois, Nobre, Tondo di Paestum, Tema 2000, Violetto di Sicilia, Violetto di Toscana, *C.c sylvestris* Creta.	Pandino et al. (2011a, b, 2013)
Cynarin (1,3-dicaffeoylquinic acid)	*Cynara cardunculus* var. *scolymus*	Panizzi and Scarpati (1954)
	Cynara scolymus Green globe, Imperial Star, Violet	Wang et al. (2003)
1,4-Dicaffeoylquinic acid	*Cynara cardunculus* L. var. *altilis* (DC)	Ramos et al. (2014)
3,4-Dicaffeoylquinic acid	*Cynara cardunculus* L. var. *altilis* (DC)	Ramos et al. (2014)
3,5-Dicaffeoylquinic acid	*Cynara cardunculus scolymus*, Violetto di Sicilia	Pandino et al. (2013)
	Cynara cardunculus (L.) subsp. *scolymus* Hayek	Garbetta et al. (2014)
1,5-Dicaffeoylquinic acid	*Cynara scolymus* L. Nobre, Tema 2000, Violetto di Sicilia, Violetto di Toscana and Terom	Pandino et al. (2011a, b, 2013), Romani et al. (2006)
	Cynara cardunculus (L.) subsp. *scolymus* Hayek	Garbetta et al. (2014)
Flavonoids		
Luteolin	*Cynara cardunculus altilis*, *Cynara cardunculus scolymus* Blanc Hye`rois, Nobre, Tempo F_1, Tondo di Paestum, Tema 2000, Violetto di Sicilia, Violetto di Toscana, Spinoso di Palermo, *C.c. sylvestris* Creta, *C.c. sylvestris* Kamaryna	Pandino et al. (2011a, b), Romani et al. (2006)
	C.c scolymus Blanca de Tudela	Abu-Reidah et al. (2013)
	Cynara cardunculus L. subsp. *scolymus* (L.)	Dranik and Chernobai (1966), Dranik et al. (1964)
	C.c scolymus Violet de Provence and Bianco di Pertosa	Fratianni et al. (2007)
Cynaroside (luteolin 7-O-β-D glucopyranoside)	*Cynara scolymus* L.	Adzet and Puigmacia (1985)
	C.c. altilis, *C.c scolymus* Blanc Hye`rois, Tempo F_1, Tondo di Paestum, Tema 2000, Violetto di Sicilia, Spinoso di Palermo, *C.c sylvestris* Creta, Kamaryna.	Shimoda et al. (2003) Pandino et al. (2011a, b)
	Cynara scolymus Green globe, Imperial Star, Violet, Violetto di Toscana	Wang et al. (2003), Romani et al. (2006)
	C.c scolymus Blanca de Tudela	Abu-Reidah et al. (2013)
	Cynara scolymus American Green globe, French Hyrious, Egyptian Baladi	Farag et al. (2013)
Luteolin 7-O-glucoronide	*C.c altilis*, *C.c scolymus* Nobre, *C.c Sylvestris* Kamaryna	Pandino et al. (2011a)

Table 2.1 Continued

Compounds	Species	References
	C.c scolymus Blanca de Tudela, Violetto di Toscana, Spinoso di Palermo	Abu-Reidah et al. (2013), Romani et al. (2006), Pandino et al. (2011a, b, 2013)
Scolymoside (luteolin 7-O-α-Lrhamnosyl (1→ 6) -β-D-glucopyranoside; luteolin-7-O-rutinoside)	*Cynara scolymus* Green globe, Imperial Star, Violet, Violetto di Toscana	Wang et al. (2003), Romani et al. (2006)
	C.c scolymus Blanc Hye`rois, Nobre, Tempo F_1, Tondo di Paestum, Tema 2000, Violetto di Sicilia, Spinoso di Palermo, *C.c sylvestris* Creta, Kamaryna.	Pandino et al. (2011a, b, 2013)
	C.c scolymus Blanca de Tudela	Abu-Reidah et al. (2013)
Luteolin acetyl hexoside	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2014)
Luteolin 7-O-malonylglucoside	*C.c scolymus* Blanc Hye`rois, Nobre Tempo F_1, Tondo di Paestum, Tema 2000, Violetto di Sicilia, Violetto di Toscana	Pandino et al. (2011a, b), Romani et al. (2006)
Apigenin	*C.c altilis*, C.c sylvestris Creta, Kamaryna, C.c scolymus Tondo di Paestum, Blanc Hyerois, Nobre, Tempo F1, Tema 2000, Violetto di Sicilia	Pandino et al. (2011a, b), Romani et al. (2006)
	C.c scolymus Blanca de Tudela	Abu-Reidah et al. (2013)
	C.c scolymus Violet de Provence and Bianco di Pertosa	Fratianni et al. (2007)
	C.c scolymus Spinoso di Palermo	Pandino et al. (2013)
Apigenin 7-O-glucoside (apigenin 7-O-β-glucopyranoside)	*C.c altilis*, *C. c sylvestris* Creta, *C.c scolymus Tondo di Paestum.*	Pandino et al. (2011a, b)
	C.c. scolymus Blanca de Tudela	Abu-Reidah et al. (2013)
	Cynara scolymus American Green globe, French Hyrious, Egyptian Baladi	Farag et al. (2013)
Apigenin 7-O-glucoronide	*C.c altilis*, *C.c scolymus* Nobre, Blanc Hyerois, Tempo F1, Tema 2000, Violetto di Sicilia *C.c sylvestris* Creta	Pandino et al. (2011a, b)
	C.c. scolymus Blanca de Tudela, Violetto di Toscana	Abu-Reidah et al. (2013)
Apigenin 7-O-rutinoside (apigenin 7-O-α-L-rhamnosyl (1 → 6) -β-D-glucopyranoside)	*C.c altilis*, *C.c scolymus* Nobre, Tondo di Paestum *C.c Sylvestris* Creta, Kamaryna.	Pandino et al. (2011a, b)
	Cynara cardunculus scolymus Green globe, Imperial Star, Violet	Wang et al. (2003)
Apigenin acetyl hexoside	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2014)
Naringenin	*Cynara scolymus* L. cv. Green globe	Sanchez-Rabaneda et al. (2003)
Naringenin 7-O-glucoside	*Cynara scolymus* L. cv. Green globe	Sanchez-Rabaneda et al. (2003)
Narirutin (naringenin 7-O-rutinoside)	*Cynara scolymus* Green globe, Imperial Star, Violet	Wang et al. (2003)
Scopoletin	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2014)
Anthocyanins	*Cynara scolymus* L. cv.''Camus'',''Green globe'', ''Le Castel'', and ''Petit Violet''	Schütz et al. (2006)
Cyanidin 3,5-diglucoside		
Cyanidin 3-sophoroside		
Cyanidin 3-glucoside		
Cyanidin 3,5-malonyldiglucoside		
Cyanidin 3-(3^{II}-malonyl) glucoside		
Delphinidin glycoside		
Peonidin 3-glucoside		
Cyanidin malonylsophoroside		
Cyanidin pentoside		
Cyanidin 3-(6^{II}-malonyl) glucoside		
Peonidin 3-(6^{II}-malonyl) glycoside		

2.7 Flavonoids

Flavonoids, together with anthocyanins, are minor constituents of artichoke, representing less than 10% of the total phenolic compounds. However, it was shown that the phenolic content of artichoke varies depending on the plant age. In general, immature heads have higher phenol contents than mature heads, where total polyphenols, detected in different cultivars of *C. carduncułus* var. *scolymus*, increased from external to internal parts (Pandino et al. 2011b).

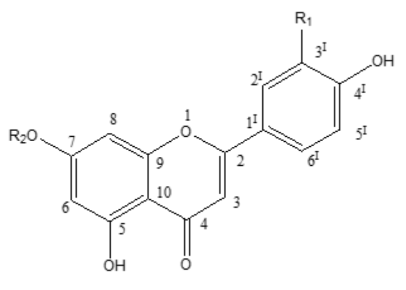
Table 2.1 shows the main flavonoids found in artichoke based on the structure of the free genin, luteolin, apigenin and naringenin and their mono and diglycosides whose chemical structure is reported in Figs. 2.2 and 2.3.

A study by Schütz et al. (2004) on globe artichoke heads led to the detection of apigenin-7-O-glucuronide, luteolin-7-O-rutinoside, and luteolin-7-O-glucoside as the main flavonoids. Pandino et al. (2010) analysed and compared the phenolic profile between capitula of wild cardoon, globe artichoke and cultivated cardoon. They reported apigenin and its 7-O-glucuronide as predominant in cultivated plant, thus confirming their potential use as source of such flavonoids. Romani et al. (2006) studied in detail all phenolic compounds present in two characteristic accessions of Tuscany (Italy), that were Violetto di Toscana and Terom, and demonstrated that flavonoids are present at higher amounts in leaves followed by heads, while stems are completely devoid of flavonoids.

Finally, the capitula of artichoke is a natural source of apigenins, one of the most important flavonoids which have several biological and pharmacological activities (see Table 2.1 for details and the following section).

Few studies focused on about the anthocyanidins group of phenols. Preliminary reports on the detection of anthocyanins in *Cynara* were those of Foury and Aubert (1977), Pifferi and Vaccari (1978) and Aubert and Foury (1981). Later on, Schütz et al. (2006) reported in detail antocyanin profile of globe artichoke inner bracts characterized by a violet colour. High performance liquid chromatography coupled with electrospray ionization mass spectrometry were used to identify

cyanidin 3,5-diglucoside, cyanidin 3-glucoside, cyanidin 3,5- malonyldiglucoside, cyanidin $3(3^{II}$-malonyl) glucoside and cyanidin 3-$(6^{II}$-malonyl) glucoside as major compound.



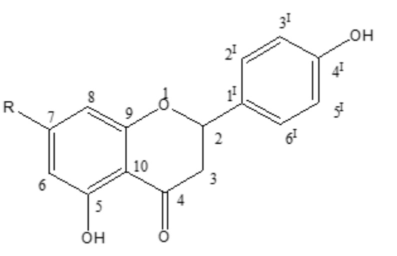
	R_1	R_2
Apigenin	H	OH
Apigenin 7 glucoside	H	X_1
Apigenin 7 glucoronide	H	X_2
Apigenin 7 rutinoside	H	X_3
Luteolin	OH	OH
Luteolin 7 glucoside	OH	X_1
Luteolin 7 glucoronide	OH	X_2
Luteolin 7 rutinoside	OH	X_3
Luteolin 7 malonyl glucoside	OH	X_4

X_1 =

X_2 =

X_3 =

X_4 =



	R
Naringenin	OH
Naringenin 7 glucoside	X_1
Naringenin 7 rutinoside	X_2

X_1 =

X_2 =

scopoletin

Fig.2.2 Chemical structures: flavonoids and flavonoid glycosides

2.7.1 Biological activity of flavonoids

Table 2.2 shows the biological activities of the isolated flavonoids including antioxidant, vasorelaxant, antibacterial, anti-hyperlipidemic, hepatoprotective and chemopreventive agent. In particular, it was shown antioxidant property for polyphenols that depends on the position and number of hydroxyl groups on the aromatic ring (Chen and Ho 1997).

According to Pandino et al. (2011a) the antioxidant capacity of the leaves was correlated with luteolin content, whereas in the floral stem, it was correlated with the content of caffeoylquinic acids. Lipid peroxidation was quantified by the production of MDA (malondialdehyde) that increases, in addition to hydroperoxide agents, when water-soluble extracts of artichoke leaves are added to primary rat hepatocyte cultures (Gebhardt 1997). Interestingly, by exposure to hydroperoxide the MDA production was inhibited, indicating a large prevention against hepatocyte necrosis of phenolic fractions of artichoke leaves. Cynaroside and its corresponding aglycon inhibit cholesterol biosynthesis in rat hepatocytes (Gebhardt 1998) also showing hypolipidemic activity. Brown and Rice-Evans (1998) also studied the dose-dependent effect of luteolin and its less active glucoside on prevention of reduction of LDL oxidation, reporting that both act as hydrogen donors and metal ion chelators.

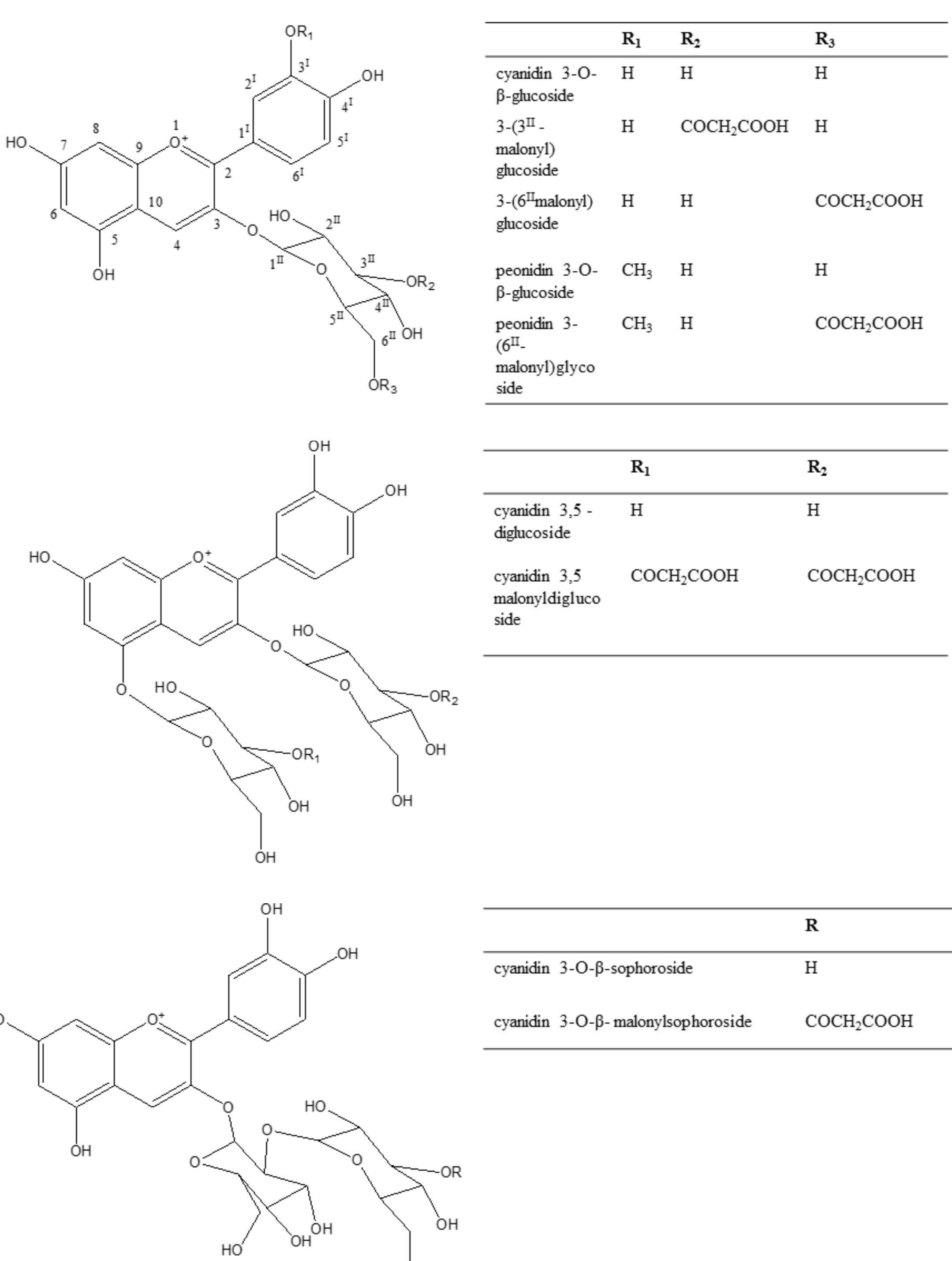
Furthermore, flavonoids, especially luteolin and its 7-O-β-D-glycopyranoside, inhibit the function of the multi-drug-resistance transporter. The binding interaction of these molecules to recombinant NBD2 (nucleotide binding domain of mouse-MDR) was investigated by Nissler et al. (2004), showing that the polar groups on C-5, C-4, and C-3^{I} are responsible for the chemical bond. In addition, the glycon part reduces this interaction compared to the aglycon compound. The higher activity of luteolin, compared to its glucoside, was also demonstrated by Gebhardt (2001) that showed biliary secretion and potent anticholestatic action of this compound.

Moreover, antimicrobial activity of artichoke leaves extract was investigated by Zhu et al. (2004). Results indicated that chlorogenic acid, 1,3-O-dicaffeoylquinic acid, luteolin-7-

rutinoside, and cynaroside have a high activity against bacteria, including *Bacillus. subtilis*, *Staphylococcus. aureus*, *Agrobacterium tumefaciens*, *Micrococcus luteus*, *Escherichia coli*, *Salmonella typhimurium*, *Psudomonas aeruginosa*, yeasts such as *Candida albicans*, *C. lusitaniae*, *Saccharomyces cerevisiae*, *S. carlsbergensis*, and the fungi *Aspergillus niger*, *Penicillium oxalicum*, *Mucor mucedo* and *Cladosporium cucumerinum*. The antimicrobial activity of the apigenin and its 7-O-glucoside against *B. subtilis* was tested by Aljancic et al. (1999) that also reported the activity only of the aglycon compound against *C. albicans* and *E. coli.* It is well known that HMG-CoA reductase is the enzyme responsible of cholesterol biosynthesis. Fritsche et al. (2002) demonstrated that chlorogenic acid, luteolin and its glucoside has a strong inhibitory effect on the enzyme, suggesting a possible use of these molecules to prevent atherosclerosis diseases. Rossoni et al. (2005) studied the vasomodulator effect of wild artichoke (*C. cardunculus*) and its main components, luteolin and apigenin, on aortic endothelial cells and on isolated rat aortic rings, showing the power of these compounds to increase the production of NO (nitric oxide-vasorelaxant factor) and to promote dose-dependent aortic relaxation.

Table 2.2 Biological activity of the isolated phenolic compounds

Compounds	Activity	References
Caffeoylquinic acids		
Chlorogenic acid (common: 3-O-caffeoylquinic acid; IUPAC: 5-O-caffeoylquinic acid) Cryptochlorogenic acid (4-O-caffeoylquinic acid)	Hepato protective, antioxidant and anticarcinogenic	Gonthier et al. (2003), Pérez-Garcı´a et al. (2000) Garbetta et al. (2014), Sato et al. (2011) Pandino et al. (2011a)
Cynarin (1,3-Dicaffeoylquinic acid)	Hepato protective	Adzet et al. (1987)
	Antioxidant	Pandino et al. (2011a), Wang et al. (2003) Pe´rez-Garcı´a et al. (2000)
	Anti-HIV	Robinson et al. (1996)
	Choleretic, anticholestatic, diuretic	Gebhardt (2000, 2001)
3,4-Dicaffeoylquinic acid	Anti-influenza viral activity	Takemura et al. (2012)
3,5-Dicaffeoylquinic acid	Antioxidant and anti-apoptotic	Zha et al. (2007), Fritsche et al. (2002)
1,5-Dicaffeoylquinic acid	Protection of astrocytes from cell death	Cao et al. (2010)
	Prevention of neuron apoptosis in Alzheimer's disease	Xiao et al. (2011)
	Anticarcinogenic	Clifford (2000)
	Antioxidant	Garbetta et al. (2014)
Flavonoids		
Luteolin	Anticholestatic, choleretic	Gebhardt (1998, 2000, 2001)
	Antioxidatant	Pandino et al. (2011a), Pérez-Garcìa et al. (2000)
	Antimicrobial activity	Zhu et al. (2004)
	Vasorelaxant	Rossoni et al. (2005)
	Inhibition of lipid peroxidation	Brown and Rice-Evans (1998)
	Inhibition of multidrug-resistant	Nissler et al. (2004)
Cynaroside (luteolin 7-O-glucoside)	Hepato protective, anticholestatic, choleretic	Adzet et al. (1987) Gebhardt (1998)
	Inhibition of lipid peroxidation	Brown and Rice-Evans (1998)
Scolymoside (luteolin 7-O-rutinoside)	Anti-hyperlipidemic	Shimoda et al. (2003)
Apigenin	Vasorelaxant	Rossoni et al. (2005)
	Antioxidant	Garbetta et al. (2014)
	Chemopreventive agent	Shukla and Gupta (2010)
	Antibacterial	Aljancic et al. (1999)



	R_1	R_2	R_3
cyanidin 3-O-β-glucoside	H	H	H
3-(3^{II}-malonyl) glucoside	H	$COCH_2COOH$	H
3-(6^{II}malonyl) glucoside	H	H	$COCH_2COOH$
peonidin 3-O-β-glucoside	CH_3	H	H
peonidin 3-(6^{II}-malonyl)glycoside	CH_3	H	$COCH_2COOH$

	R_1	R_2
cyanidin 3,5 - diglucoside	H	H
cyanidin 3,5 malonyldiglucoside	$COCH_2COOH$	$COCH_2COOH$

	R
cyanidin 3-O-β-sophoroside	H
cyanidin 3-O-β- malonylsophoroside	$COCH_2COOH$

Fig. 2.3 Chemical structures: anthocyanin glycosides

2.8 Triterpenes and sesquiterpenes

Triterpenes (Figs. 2.4 and 2.5) and sesquiterpene lactones (Fig. 2.6) were found to be the major class of lipophilic components of cultivated cardoon (Table 2.3). Sesquiterpenes are mainly concentrated in the leaves and present in low amounts in the stalks and capitula, in contrast

triterpenes are present at lower amounts in the leaves (Ramos et al. 2013). The authors also identified cynaropicrin as predominant sesquiterpene in the leaves. Cyanoropicrin was isolated for the first time by Suchy et al. (1960) as the bitter principle of artichoke. Recently, Ramos et al. (2013) also reported for the first time in *C. cardunculus* L. var. *altilis* (DC) the presence of the following components: deacylcynaropicrin, lupenyl acetate and ψ-taraxasteryl acetate, the latter triterpene being the most abundant one.

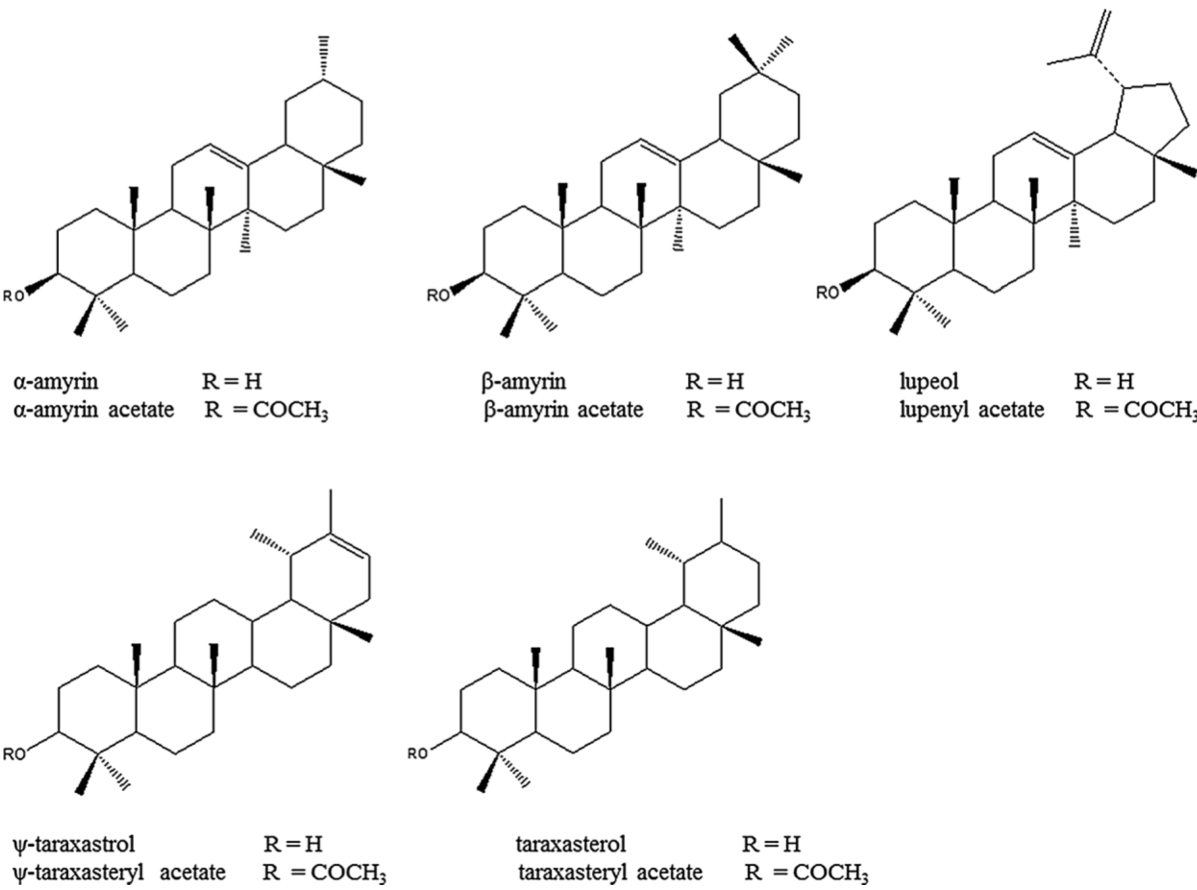
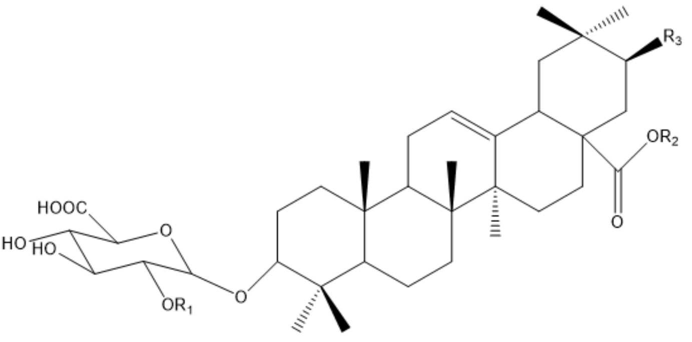


Fig. 2.4 Chemical structures: triterpenes

	R1	R2	R_3	R_4
Cynarasaponin A	Arabinose	Glucose	H	CH_3
Cynarasaponin B	Arabinose	H	H	H
Cynarasaponin C	H	Glucose	H	CH_3
Cynarasaponin D	Arabinose	Glucose	H	CH_2OH
Cynarasaponin E	H	CH_3	H	CH_2OH
Cynarasaponin F	Arabinose	H	OH	H
Cynarasaponin G	Arabinose	Glucose	OH	H



	R1	R2	R_3
Cynarasaponin H	Arabinose	Glucose	H
Cynarasaponin I	Arabinose	H	OH
Cynarasaponin J	Arabinose	Glucose	OH

Fig.2.5 Chemical structures: saponins, triterpene glycosides

2.8.1 Biological activity of triterpenes and sesquiterpenes

Triterpenes and sesquiterpenes (cynaropicrin, cynarascolosides A, B, and C) were isolated by Shimoda et al. (2003) as the active artichoke components. Particularly, it was demonstrated that the oxygen at position 3 and 8, and the exo-methylene group are essential for anti-hyperlipidemic activity (Fig. 2.6). The most abundant sesquiterpene in artichoke is cynaropicrin that showed several biological effects (Table 2.4), such as inhibition of contraction of rabbit isolated thoracic aorta (Hay et al. 1994) and antihyperlipidemic activity (Shimoda et al. 2003). Similar activity has been found for aguerin B and grosheimin. Interestingly, a cytotoxic activity of cynaropicrin and aguerin B against cultured human tumour cell lines was later showed by Choi et al. (2005), though the two sesquiterpene lactones were not isolated from artichoke, but from a different plant species (i.e. *Saussurea calcicola* Nakai). In this respect, Tanaka et al. (2013) tested several plants extracts in order to study their inhibitory effect on the nuclear factor Kappa B (NF-kB), which is one of the most important transcription factors activated in keratinocytes by UV irradiation, which can lead to cancer onset. *C. scolymus* L. was found as the most active plant. After isolation of bioactive compounds, it was reported that cynaropicrin had the greatest suppressive effect, suggesting that such metabolite can be considered as a protector of skin aging, particularly against epidermal hyperproliferation and melanocyte proliferation induced by UVB (Table 2.4). Yasukawa et al. (2010) reported an inhibitory effect of triterpenes and their acetates on the inflammation induced by TPA (12-O-tetradecanoylphorbol-13-acetate-tumour promoter) on mouse skin. The active constituents of artichoke flower extracts (*C. cardunculus* L.) were separated, tested and compared to indomethacin: α and β amyrin, taraxasterol, ψ-taraxasterol and their acetates exerted anti-inflammatory activity, with α and β amyrin more active than indomethacin. It is interesting to underline that the anticarcinogenic activity of taraxasterol against TPA on mouse skin had already been tested (Yasukawa et al. 1996).

Cynarascoloside A
Cynarascoloside B
Cynarascoloside C

β-cubebene cynaratriol grosheimin

R = cynaropicrin
R = OH deacylcynaropicrin
R = aguerin B

Fig. 2.6 Chemical structures: sesquiterpene and sesquiterpene glycosides

In addition, α and β amyrin showed antiinflammatory properties in mouse induced colitis (Vitor et al. 2009), with both of these triterpenes diminishing interleukin (IL)-1β, cytokines and COX-2 levels. In addition, β-amyrin acetate was more active in decreasing the secretion of tumor necrosis factor (TNF-α) even at low concentration (Ding et al. 2009) and also showed inhibitory activity against human breast and ovarian cancer cell line. Triterpenoid compounds were also extracted from involucres bracts of *C. cardunculus* L. by Krimkova et al. (2004) in order to

verify the antimutagenic activity of cynarasaponins against acridine orange (AO) induced damage of chloroplast DNA in the green alga *Euglena gracilis*.

Table 2.3 Terpenoid compounds isolated from artichoke

Compounds	Species	References
Sesquiterpenes		
Aguerin A, B	*Cynara scolymus* L.	Shimoda et al. (2003)
Grosheimin	*C. cardunculus* L. var. *altilis* (DC) *C. cardunculus* L. var. *scolymus*	Shimoda et al. (2003)
Cynaropicrin	*Cynara scolymus* L. *Cynara cardunculus* L.	Tanaka et al. (2013) Shimoda et al. (2003)
Deacylcynaropicrin	*Cynara cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)
Cynarascoloside A, B, C	*Cynara scolymus* American Green globe, French Hyrious, Egyptian Baladi	Farag et al. (2013)
β-Cubebene	*Cynara scolymus* L.	Hadaruga et al. (2009)
Guaianolides (sesquiterpene lactones)		
11-H-13 methylsulfonylgrosheimin 8-deoxy-11-hydroxy-13 chlorogrosheimin 8-deoxy-11,13-dihydroxygrosheimin 8-epigrosheimin Sibthorpine	*Cynara scolymus* L.	Barbetti et al. (1993)
Triterpenes		
Cynarasaponins	*C. cardunculus* L. *Cynara scolymus* American Green globe, French Hyrious, Egyptian Baladi	Krimkova et al. (2004) Farag et al. (2013)
α and β amyrin	*C. cardunculus* L. var. *altilis* (DC)	Akihisa et al. (1996) Ramos et al. (2013)
α and β amyrin acetate	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)
Lupeol	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)
Lupenyl acetate	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)
ψ-Taraxasterol	*C. cardunculus* L. var. *altilis* (DC)	Akihisa et al. (1996)
Taraxasterol	*C. cardunculus* L. var. *altilis* (DC)	Akihisa et al. (1996) Yasukawa et al. (1996)
ψ-Taraxasteryl acetate	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)
Taraxasteryl acetate	*C. cardunculus* L. var. *altilis* (DC)	Ramos et al. (2013)

Table 2.4 Biological activity of the isolated terpenoid compounds

Compounds	Activity	References
Sesquiterpenes		
Grosheimin	Anti-hyperlipidemic	Shimoda et al. (2003)
	Cytotoxic	Choi et al. (2005)
	Anti-hyperlipidemic	Shimoda et al. (2003)
Cynaropicrin	Antiphotoaging agent;	Tanaka et al. (2013)
	Anti-hyperlipidemic	Shimoda et al. (2003) Fritsche et al. (2002)
	Inhibition of thoracic aorta contraction	Hay et al. (1994)
	Cytotoxic	Choi et al. (2005)
Triterpenes		
Cynarasaponins	Antimutagenic	Krimkova et al. (2004)
α and β amyrin	Anti-inflammatory	Akihisa et al. (1996) Yasukawa et al. (2010) Vitor et al. (2009)
	Anticarcinogenic	Ding et al. (2009)
α and β amyrin acetate	Anti-inflammatory	Yasukawa et al. (2010)
ψ-Taraxasterol	Anti-inflammatory	Akihisa et al. (1996) Yasukawa et al. (2010)
Taraxasterol	Anti-inflammatory Inhibitor of skin tumor Inhibitor of skin tumor	Akihisa et al. (1996) Yasukawa et al. (1996)
ψ-Taraxasteryl acetate	Anti-inflammatory	Yasukawa et al. (1996)
Taraxasteryl acetate	Anti-inflammatory	Yasukawa et al. (1996)

2.9 Inulin and its biological activity

Inulin is a polysaccharide belonging to the family of fructans whose chemical structure is based on linked fructose units β-(1 → 2) that end with a α-(1 → 2) glucose (Fig. 2.7). It has been detected in the edible artichoke heads in a concentration ranging from 18.9 to 36.2% of dry weight (Lattanzio et al. 2009). Inulin is a polydisperse polymer with different degree of polymerization that is expressed by the general formula GFn (G = glucose; F = fructose; n = number of fructose units) with n ranging from 4 to 100. It is important to determine its degree of polymerization because it influences several properties, such as solubility (high degrees are less water-soluble), thermal stability (high degrees are more stable), sweetness and prebiotic

activity. Inulin belongs to non-digestible oligosaccharides (NDO), because its special linkages cannot be degraded by human digestive system. Therefore, the assumed dose does not increase the level of sugar blood because the molecule is not absorbed in the gastrointestinal tract where it acts by increasing water flow (osmotic process), consequently it is fermented by microflora. For this reason, artichoke can be considered as a functional food composed by inulin, a most of important dietary fibre (Robenfroid 1999). Gibson et al. (1995) demonstrated in vivo that inulin administration selectively stimulates the *Bifidobacterium* in the human colon, also reporting an increase in daily stool output when inulin was introduced into the diet. Van Loo et al. (1999) also studied the effect of inulin on the microflora colonies. In particular, he demonstrated its prebiotic effect and how it enhances bacterial biomass with a consequent increase in faecal output. This was confirmed by the production of short-chain fatty acids (final products of the fermentation process), especially acetate and butyrate. A decreased concentration of tumour-promoting substances, such as ammonia, for fructooligosaccharide administration was also reported (Gallaher et al. 1996).

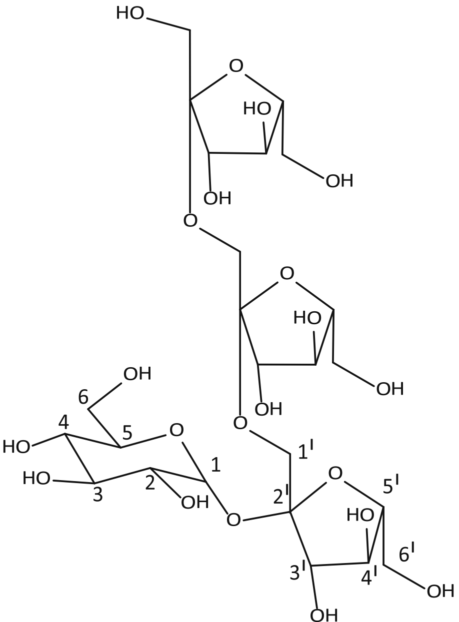


Fig. 2.7 Chemical structure: inulin

Stewart et al. (2008) tested whether the chain length could influence the fermentation of inulin and demonstrated that short chains are more rapidly fermented than long chains, while Tarrega et al. (2010) and Meyer et al. (2011) suggested a mixture of short and long chains inulin in order to improve its use in commercial prebiotic products and prolong its prebiotic activity in the colon. Further demonstrated biological effects include a reduction of serum triglycerides in men volunteers after inulin administration (Causey et al. 2000). Finally, it was shown both by experimental data and human observations that inulin-type fructans are able to reduce the colon cancer risk (Pool-Zobel et al. 2002; Pool-Zobel 2005).

2.10 Comparative analysis of phytochemistry and pharmacology studies

A comparative analysis of the studies reporting on qualitative and/or quantitative analysis, as well as biological activity of metabolites found in *C. cardunculus* s. lat. was carried out. Starting from the 100 references cited in the present review, 81 studies were selected, whereas 19 items were discarded since reporting not relevant information. These included an international on-line database on crop production (FAO 2013), 3 books of general interest about medicinal plants (Schauenberg and Paris 1977; Chevallier 1996) and herbs (Fleming 1998), 2 monographs on the Cilento National Park (SouthWest Italy) dealing with dyeing plants (De Falco and di Novella 2011) and multifunctional sustainable agriculture (Amato et al. 2011), respectively, a review (Verpoorte et al. 2008) and a methodological paper (Incerti et al. 2013) on plant metabolomics, and 11 book chapters and research articles reporting on artichoke horticulture (Bianco 1990, Cantore and Boari 2009), breeding (Lanteri and Portis 2008), phenology (Virdis et al. 2009, 2014; Archontoulis et al. 2010), origin and evolution (Rottenberg and Zohary 1996; Pignone and Sonnante 2004, 2009), and population genetics (Portis et al. 2005; Sonnante et al. 2008). The 81 selected papers were further subdivided into two datasets of 26 and 55 items,

corresponding to phytochemistry and health effect studies, respectively, based on the presence of information on the biological activity of targeted metabolites.

2.10.1 Comparative analysis of studies on artichoke metabolites identification

In the phytochemistry comparative analysis, we considered the number of experiments reporting evidence of extraction, purification, and characterization of metabolites from specific plant parts of the three *C. cardunculus* subspecies (i.e. *scolymus*, *altilis*, *sylvestris*). We have also taken into account the occurrences of different analytical techniques, in order to provide a view of the methods most and least used in previous studies, which could be useful for scientist working in the field. Many of the 26 studies reported experiments on different plant parts (e.g. Ramos et al. 2013) and/or class of compounds (e.g. Farag et al. 2013), so that a total of 104 reports of artichoke and cardoon metabolites were reviewed. Polyphenols are by far the most often reported compounds (Table 2.5), with very similar occurrences of both caffeoylquinic acids and flavonoids, which in most studies were searched together. Such compounds were mostly searched in artichoke leaves and in the edible heads and bracts, and found in all plant parts with the exception of flowers and roots. Anthocyanins and the oligofructan inulin were much rarely searched, and selectively found in edible (heads, bracts) and discarded (stems, leaves, and roots) parts of the plant, respectively. Sesquiterpenes were rarely searched, but ubiquitarily found, as well as triterpenes and fatty acids. Expectedly, the most studied plant parts are the edible head and the leaves, with a total of 27 and 28 reports, respectively, that taken together correspond to over 50% of the total reports. Bracts (16 reports), receptacles (13) and stems (11) were less explored, while flowers (5 reports, only 2 of globe artichoke) and roots (2 reports) were likely overlooked. The analytical tools used in the reviewed studies (Table 2.5) vary over a wide range, resembling the historical advancement in the field of analytical chemistry. From traditional precipitation, purification, and crystallization methods, as in the

classic pioneeristic study of Panizzi and Scarpati (1954), or the simple Column Chromatography followed by TLC, as in Dranik and Chernobai (1966) the instrumental evolution allowed the recent application of Ultra High Performance Liquid Chromatography techniques for compounds separation, coupled to advanced tools and Mass Spectrometry for specific molecule detection, that was recently used to quickly characterize different class of compounds in globe artichoke (Farag et al. 2013). However, most studies were based on HPLC–MS, used to isolate polyphenols (Table 2.5). Terpenes and fatty acids were mostly searched by GC–MS, as in Ramos et al. (2013), while inulin was found in leaves, stems, and roots of globe artichoke by Raccuia et al. (2004) in the unique application of high-performance anion-exchange chromatography (Table 2.5). Interestingly, a high throughput technique such as NMR spectroscopy, widely used to characterize plant metabolomics coupled with multivariate statistics (e.g. Verpoorte et al. 2008; Incerti et al. 2013) was rarely used in the case of artichoke, and only once as a metabolomics tool (de Falco et al. 2015). In two further cases a metabolomic analysis of artichoke was tentatively proposed based on a multivariate approach: both Farag et al. (2013) and Hadarugaa et al. (2009) used the Principal Component Analysis method, in order to analyse UHPLC–MS data from three cultivars of globe artichoke, and GC–MS data from four species of Asteraceae (including globe artichoke), respectively.

Table 2.5 Comparative analyisis of 25 papers on artichoke and cardoon phytochemistry

	Class of compounds							
	Caffeoylquinic acids	**Flavonoids**	**Anthocyanins**	**Triterpenes**	**Sesquiterpenes**	**Fatty acids**	**Inulin**	**Total**
C. cardunculus parts								
Heads	7 (2) [1]	7 (2)	4	(1)	(1) [1]	(1)		18 (7) [2]
Bracts	5 (1)	5 (1)	1	(1)	(1)	(1)		11 (5)
Flower			1	(1)	1 (1)	(1)		2 (3)
Receptacle	4 (1)	4 (1)		(1)	(1)	(1)		8 (5)
Stems	3	3		(1)	1 (1)	(1)	1	8 (3)
Leaves	9 (1)	7 (1)		1 (1)	4 (1)	1 (1)	1	23 (5)
Roots					1		1	2
Juice, pomace	1	1						2
Total	29 (5) [1]	27 (5)	6	1 (6)	7 (6) [1]	1 (6)	3	74 (28) [2]
Analytical methods*								
CC	2	2	3	6	7	6		26
TLC	2	3						5
Vis, UV, AA	4	4						8
MS	20	20						40
HPLC	30	29						59
HPLC–DAD– ESI–MS	2	2	3					7
UHPLC–QTOF– MS	2	2		1	1	1		7
IR					1			1
NMR				6	7	6		19
GC–MS				6	14	6		26
HPAEC							3	3
Total	62	62	6	19	30	19	3	201

Table shows the number of reports on extraction, purification and characterization of metabolites from *C. cardunculus* subsp. *scolymus*, according to plant parts and class of compounds. Values in round and square brackets refer to experiments on *C. cardunculus* subsp. *altilis*, and *C. cardunculus* subsp. *sylvestris*, respectively. Occurrences of analytical methods in the experiments are also shown.

Please note that number of reports does not correspond to the number of papers, the since one paper can report experiments on different plant parts and/or class of compounds.

*Methods abbreviations: CC column chromatography, TLC thin layer chromatography, Vis, UV, AA visible, ultra-violet, and atomic absorption spectroscopy, MS mass spectrometry, HPLC high performance liquid chromatography, DAD–ESI diode array detection– electrospray ionisation, UHPLC–QTOF ultra high performance liquid chromatography–quadrupole time-of-flight, IR infra-red spectroscopy, NMR nuclear magnetic resonance spectroscopy, GC gas chromatography, HPAEC high-performance anion-exchange chromatography

2.10.2 Comparative analysis of studies of metabolites health effects

We considered the number of experiments, from the 55 reviewed papers, reporting evidence of biological activity of *C. cardunculus* metabolites, even when derived from different plants, also taking into account the type of effect, the active principles, the target biological system in vitro and/or in vivo in the original studies. In this respect, a proper meta-analysis of the results from previous studies on health effects by artichoke metabolites was avoided, due to the high number of different compounds and tested biological activities reported in the literature (Tables 2.6 and 2.7), as compared to the limited total number of reports. Indeed, also considering the methodological differences among different previous studies, the number of available outcomes on specific effects was limited, preventing from consistent and informative result comparisons (Table 2.7). On the other hand, we considered the occurrences of different methods used to assess the biological effects of artichoke metabolites, in order to highlight the most used techniques, as well as the least or not yet tested approaches, which could help to immediately detect the specific topics needing to be addressed by further studies. A total of 91 reports of biological activity of artichoke and cardoon metabolites were reviewed. Thirty-nine experiments were performed using active principles extracted from *C. cardunculus*, mostly from the *scolymus* subspecies (26 cases), but also from the *altilis* subspecies (2), while in 11 cases the subspecies was not indicated (Table 2.6). In 26 cases the active compounds, known for artichoke, were not directly derived from plants, but acquired from commercials. In the remaining 26 experiments, the metabolites were derived from 21 different plant species, all belonging to the Asteraceae family, with the exceptions of two species from Anacardiaceae and Rubiaceae, respectively (Table 2.6). When arranged according to the type of biological activity and the related assessment methods (Table 2.7) the 91 reports were very variable, with 17 different targeted activities.

Table 2.6 Comparative analyisis of 55 papers on artichoke and cardoon pharmacology

Source taxa	Caffeoylquinic acids	Flavonoids	Sesquiterpenes	Triterpenes	Fatty acids	Inulin	Total
None (compounds acquired from commercial)	14	4		1		7	26
Asteraceae							
C. cardunculus L. s. lat.	3	5		3			11
C. cardunculus L. subsp. *scolymus* (L.) Hegi	10	9	3	2	1	1	26
C. cardunculus L. subsp. *altilis* DC.	1	1					2
Achillea atrata L.		1	1				2
Achyrocline alata (Kunth) DC.	1						1
Arctium lappa L.				1			1
Baccharis genistelloides Pers.	1						1
Calendula officinalis L.				1			1
Carthamus tinctorius L.				1			1
Centaurea solstitialis L.			1				1
Chrysanthemum morifolium Ramat.				2			2
Cichorium intybus L.						3	3
Cirsium nipponicum Makino				1			1
Cirsium tanakae Matsum.				1			1
Cosmos bipinnatus Cav.				1			1
Helianthus annuus L.				1			1
Helianthus debilis Nutt.				1			1
Matricaria matricarioides Porter.				1			1
Saussurea calcicola Nakai			1				1
Silybum marianum Gaerm.				1			1
Taraxacum officinale Weber.				1			1
Taraxacum platycarpum Dahlst.				1			1
Anacardiaceae							
Toxicodendron sylvestre Kuntze				1			1
Rubiaceae							
Gardenia jasminoides J.Ellis	2						2
Total	32	20	6	21	1	11	91

Table shows the number of reports on biological activity of metabolites found in *C. cardunculus* subsp. *scolymus*, according to class of compounds and plant taxa used as source

The most investigated (35 reported experiments, corresponding to over 38% of the total) was certainly the antioxydant activity of polyphenols, both caffeoylquinic acids and flavonoids. However, the methodological approaches followed in these experiments were highly variable,

with 27 types of in vitro assays based on 13 different targets-i.e. malondialdehyde (MDA), hydrogen peroxyde (H_2O_2), lactate dehydrogenase (LDH), tetrazolium (MTT), dichloro-dihydro-fluorescein diacetate (DCFH), Rancimat, phorbol-12-myristate-13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (FMLP), fluorescence recovery after photobleaching (FRAP), trolox-equivalent antioxidant capacity (TEAC), 2,2- diphenyl-1-picrylhydrazyl (DPPH), ferric thiocyanate (FTC), low-density lipoprotein (LDL). In addition, 7 in vitro bioassays were performed on 5 different human and rat cell lines (Table 2.7), and one in vivo test on rats. Interestingly, notwithstanding such wide experimental background, a systematic review on the antioxidant activity of artichoke polyphenols has not yet been compiled, while, for example, nine reviews on nutrition, metabolism and functional food properties of artichoke metabolites were found, with only three experimental studies, two on inulin and oligofructans and one on caffeoylquinic acids (Table 2.7). Other well documented biological activities of polyphenols include anticholestatic, choleretic, and hepatoprotective functions (eight reports, both in vitro and in vivo) and anti-microbial activity (seven reports). Among the most relevant biological functions, antitumour activity was investigated in vitro for triterpenes (Yasukawa et al. 2010) and sesquiterpenes (Choi et al. 2005) and in vivo for oligofructans (Gallaher et al. 1996). However, no recent papers were found following those promising outcomes. Considering the most advanced experiments on possible uses of artichoke metabolites, clinical trials on human have been successfully presented for the anti-hyperlipidemic (Causey et al. 2000) and colon microfloraenhancing (Gibson et al. 1995) activities of inulin and oligofructans (Table 2.7).

Table 2.7 Comparative analysis of 55 papers on artichoke and cardoon pharmacology

Biological activity and experimental method	Caffeoyl quinic acids	Flavonoids	Sesqui-terpenes	Tri-terpenes	Fatty acids	Inulin	Total
Anti skin photo-ageing							
In vivo Bioassay (mices)			1				1
Anticholestatic, choleretic, hepatoptotective							
In vitro CCl4 assay, bioassay (rat hepatocytes)	2	5					7
In vivo Bioassay (rats)	1						1
Anti-hyperlipidemic (cholesterol, triglycerides)							
In vitro Rancimat assay	1	1					2
In vivo Bioassay (olive oil-loaded mices)			1				1
Human Clinical trial						1	1
Anti-inflammatory							
In vitro Bioassay (rat macrophage cell line)				1			1
In vivo Bioassay (mices)				3			3
Antimicrobial							
In vitro DPPH assay, bioassays (bacteria, yeasts, fungi)	2	3	1				6
Antimutagenic							
In vitro Bioassay (Euglena gracilis)				1			1
Antioxydant							
In vitro MDA, H2O2, LDH, MTT, DCFH, Rancimat assays	9	5					14
In vitro PMA, FMLP, FRAP, TEAC, DPPH, FTC, LDL assays	6	7					13
In vitro Bioassays (Caco-2, human neutrophil and intestinal, rat hepatocytes and astrocytes cell lines)	6	1					7
In vivo Intestinal ischemia–reperfusion model (rats)	1						1
Anti-tumoral							
In vitro Bioassay (human tumor cell line)			1	1			1
In vivo Bioassay (mices, rats)				1		1	3
Review		1				2	3
Anti-viral (HIV)							
In vitro Bioassay (T cell lines, tissue culture)	1						1
Anti-viral (IAV)							
In vivo Bioassay (mices)	1						1
Inhibition of multi-drug-resistence transporter							
In vitro Tryptophan fluorescence-quenching assay		1					1
Inhibition of smooth muscle contractility							
Ex vivo Bioassay (rabbit aorta rings)			1				1
Lipoxygenase inhibitor							
In vitro Lipoxygenase assay	2						2
Neuroprotective							
In vitro Bioassay (rat cultured cortical neurons)	1						1
Nutrition, metabolism and functional food properties							
In vitro Rheological and sensory assay, batch fermentation						1	1

Table 2.7 Continued

Biological activity and experimental method		Caffeoyl-quinic acids	Flavonoids	Sesqui-terpenes	Tri-terpenes	Fatty acids	Inulin	Total
In vivo	Bioassay (rats)	1					1	2
	Review	2	1	1	1	1	3	9
Prevention of endothelial and vasomotor function loss								
In vitro	Bioassays (rat aortic endothelial cell line)		1					1
In vitro	DNA fragmentation analysis, Caspase-3 assay	1						1
Protection from colitis, stimulation of microflora								
In vitro	Bioassay (bifidumbacterium, mixed colonic bacteria)						1	1
In vivo	Bioassay (mices, rats)				1		1	2
Human	Clinical trial						1	1
	Total	37	26	6	9	1	12	91

Table shows the number of reports on biological activity of metabolites found in *C. cardunculus* subsp. *scolymus*, according to type of activity, experimental method, and class of compounds

2.11 Conclusion

Artichoke is a food plant known since ancient times as medicine and component of Mediterranean diet. In fact, it was known in Europe since the 15th century and later on brought to the Americas by European immigrants. Production of artichokes amounts to 1,793,015 tones year^{-1}, more than 60% of which in Europe (FAO 2013) with Italy as the first world producer, followed by China, where this species has acquired commercial importance in the last two decades. To overcome the problems related to genetic variability and infections, and to reduce the labour requirements for its reproduction, in vitro culture or seed propagation are increasingly used. Seed production occurs through free pollination thus producing a high genetic, phenotypic and phenological variability. The capitulum or heads, constituting the immature inflorescence, are the edible part of the plant and constitute the 15–20% of the total biomass. All parts of the plant have been shown to be a rich source of nutraceutical that belong to different classes of organic compounds such as polyphenols, fructans, flavonoids, anthocyanins, triterpenes and sesquiterpenes. These compounds were isolated by classical procedures based on chromatographic purification steps, followed by structure elucidation studies. Recent high throughput approaches by UHPLC– MS and NMR spectroscopy, followed

by principal component analysis, were recently used to characterize the artichoke metabolome of different cultivars (Farag et al. 2013; de Falco et al. 2015), being these rapid and efficient methodologies. The main biological activities attributed to the artichoke metabolites were hepatoprotection (Adzet et al. 1987), choleretic (Preziosi et al. 1959), lipid-lowering (Gebhardt 1998), and colon cancer protection (Pool-Zobel 2005). However, further studies are needed to fully clarify the biological activity of the artichoke metabolites and their mechanism of action.

References

Abu-Reidah IM, Arráez-Román D, Segura-Carretero A et al (2013) Extensive characterisation of bioactive phenolic constituents from globe artichoke (*Cynara scolymus* L.) by HPLC-DAD-ESI-QTOF-MS. Food Chem 141(3):2269–2277

Adzet T, Camarasa J, Laguna JC (1987) Hepatoprotective activity of polyphenolic compounds in isolated rat hepatocytes from *Cynara scolymus* against CCl_4 toxicity: in isolated rat hepatocytes. J Nat Prod 50 (4): 612–617

Adzet T, Puigmacia M (1985) High-performance liquid chromatography of caffeoylquinic acid derivatives of *Cynara scolymus* L. leaves. J Chromatogr 348(2):447–453

Akihisa T, Yasukawa K, Oinuma H et al (1996) Triterpene alcohols from the flowers of Compositae and their anti-inflammatory effects. Phytochemistry 43(6):1255–1260

Aljancić I, Vajs V, Menković N et al (1999) Flavones and Sesquiterpene Lactones from *Achillea atrata* subsp. multifida: Antimicrobial Activity. J Nat Prod 62:909–911

Amato M, D'Orilia F, Gay V et al (2011) Multifunctional agriculture and sustainability in the Cilento Geopark. In: Dolven JK, Ramsay T, Rangnes K (eds) Proceedings of the 10th European Geoparks Conference. European Geoparks Network, Porsgrunn, Norway, pp 41–48

Archontoulis SV, Struik PC, Vos J et al (2010) Phenological growth stages of *Cynara cardunculus*: Codification and description according to the BBCH scale. Ann Appl Biol 156: 253–270

Aubert S, Foury C (1981) Couleur et pigmentation anthocyanique de l'artichaut (*Cynara scolymus* L). In: Marzi V, Lattanzio V (eds) Studi sul Carciofo. Laterza, Bari. pp 57–76

Barbetti P, Chiappini I, Fardella G et al (1993) Grosulfeimin and new related guaianolides from *Cynara scolymus* L. Nat Prod Lett 3:21–30

Bianco VV (1990) Carciofo (*Cynara scolymus* L.). In: Bianco VV, Rimpini F (eds) Orticoltura. Patron, Bologna

Brown JE, Rice-Evans C (1998) Luteolin-rich artichoke extract protects low-density lipoprotein from oxidation in Vitro. Free Radical Res 29:247–255

Cantore V, Boari F (2009) Irrigazione e Salinità. In: N. Calabrese (ed) Il carciofo e il cardo. Script, Bologna, pp 190-197

Cao X, Xiao H, Zhang Y et al (2010) 1,5 Dicaffeoylquinic acid-mediated glutathione synthesis through activation of Nrf2 protects against OGD/reperfusion-induced oxidative stress in astrocytes. Brain Res 1347:142–148

Causey BSJL, Feirtag JM, Gahaher DD et al (2000) Effects of dietary inulin on serum lipids, blood glucose and the gastrointestinal environment in hypercholesterolemic men. NutrRes 20(2):19l–201

Chen JH and Ho CT (1997) Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds. J Agric Food Chem 45:2374−2378

Chevallier A (1996) The Encyclopedia of Medicinal Plants. DK Publishing, New York, pp 96–97

Choi SZ, Choi SU, Lee KR (2005) Cytotoxic sesquiterpene lactones from *Saussurea calcicola*. Arch Pharm Res 28:1142−1146

Christaki E, Bonos E, Florou-Paneri PC (2012) Nutritional and functional properties of *Cynara* crops (globe artichoke and cardoon) and their potential applications: A review. Int J Appl Sci Technol 2: 64–70

Clifford M (2000) Chlorogenic acids and other cinnamates – nature, occurrence, dietary burden, absorption and metabolism. J Sci Food Agric 80:1033–1043

de Falco B, Incerti G, Pepe R et al (2015) Metabolomic fingerprinting of artichoke, *Cynara cardunculus*, using nuclear magnetic resonance and multivariate data analysis. Proceedings of the Phytochemical Society of Europe (PSE), Future Trends in Phytochemistry in the Global Era of Agri-Food and Health II. A Young Scientist Meeting, 27-30/4/2015, Murcia, Spain. I.S.B.N. 978-0-9565472-6-2 pp 72-73

De Falco E, di Novella N (2011) Guida alle piante tintorie del Cilento e Vallo di Diano. MIdA, Milano

Ding Y, Nguyen HT, Kim SI et al (2009) The regulation of inflammatory cytokine secretion in macrophage cell line by the chemical constituents of *Rhus sylvestris*. Bioorg Med Chem Lett 19:3607–3610

Dranik LI, Chernobai VT (1966) A new flavonoid isolated from the leaves of *Cynara scolymus* L. Chem Nat Compd 2(1):16–20

Dranik LI, Chernobai VT, Kolesnikov DG (1964) Polyphenolic compounds of *Cynara scolymus*. Med Prom SSSR 18:23

Durazzo A, Foddai MS, Temperini A et al (2013) Antioxidant Properties of Seeds from Lines of Artichoke, Cultivated Cardoon and Wild Cardoon. Antioxidants 2:52–61.

Falleh H, Ksouri R, Chaieb K et al (2008) Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. C R Biol 331:372–379

FAO (2013) Faostat, Crop production. Food and Agriculture Organization of the United Nations. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. cited 21 May 2013.

Farag MA, El-Ahmady SH, Elian FS et al (2013) Metabolomics driven analysis of artichoke leaf and its commercial products via UHPLC–q-TOF-MS and chemometrics. Phytochemistry 95:177–187

Fleming T (ed) (1998) PDR for Herbal Medicines. Medical Economics Company, Montvale, pp 793–794

Foury C, Aubert S (1977) Observations preliminaries sur la presence et la repartition de pigments anthocyaniques dans un mutant d'artichaut (*Cynara scolymus* L.) a fleur blanche. Ann Amélior Plant 27: 603–612

Fratianni F, Tucci M, De Palma M, et al (2007) Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynara cardunculus* L. var. *scolymus* (L.) Fiori). Food Chem 104:1282–1286

Fritsche J, Beindorff CM, Dachtler M et al (2002) Isolation, characterization and determination of minor artichoke (*Cynara scolymus* L.) leaf extract compounds. Eur Food Res Technol 215:149–157

Gallaher DD, Stallings WH Blessing LL et al (1996) Probiotics, cecal microflora, and aberrant cryps in the rat colon. J Nutr 126:1362–1371

Garbetta A, Capotorto I, Cardinali A et al (2014) Antioxidant activity induced by main polyphenols present in edible artichoke heads: influence of in vitro gastro–intestinal digestion. J Funct Food 10:456–464

Gebhardt R (1997) Antioxidative and protective properties of extracts from leaves of the artichoke (*Cynara scolymus* L.) against hydroperoxide-induced oxidative stress in cultured rat hepatocytes. Toxicol Appl Pharmacol 144(2):279–286

Gebhardt R (1998) Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (*Cynara scolymus* L.) extracts. J Pharmacol Exp Ther 286:1122–1128

Gebhardt R (2000) Choleretic and anticholestatic activities of flavonoids of artichoke (*Cynara cardunculus* L. subsp. *scolymus* L. Hayek). Acta Hortic 681:429–436

Gebhardt R (2001) Anticholestatic activity of flavonoids from artichoke (*Cynara scolymus* L.) and of their metabolites. Med Sci Monit 7:316–320

Gibson GR, Beatty ER, Wang X (1995) Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108:975–982

Gonthier MP, Verny MA, Besson C et al (2003) Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr 133:1853–1859

Hădărugăa NG, Hădărugăb DI, Tatuc C et al (2009) Multivariate analysis (PCA) in Compositae biocompounds class. J Agroaliment Proc Technol 15(2): 201–210

Hay AJ, Hamburger M, Hostettmann K et al (1994) Toxic inhibition of smooth muscle contractility by plant-derived sesquiterpenes caused by their chemically reactive alpha-methylenebutyrolactone functions. Br J Pharmacol 112(1):9–12

Incerti G, Romano A, Termolino P et al (2013) Metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis as a tool for biodiversity informatics: A case study on the classification of *Rosa* x *damascena*. Plant Biosys 147(4):947–954

Krimkova L, Mučaji P, Nagy M et al (2004) Triterpenoid cynarasaponins from *Cynara cardunculus* L. reduce chemically induced mutagenesis in vitro. Phytomedicine 11:673–678

Lanteri S, Portis E (2008) Globe Artichoke and Cardoon. In: Prohens J, Nuez F (eds) Vegetables. Handbook of plant breeding vol 1. Springer, New York, pp 49–74.

Lattanzio V, Kroon PA, Linsalata V et al (2009) Globe artichoke: A functional food and source of nutraceutical ingredients. J Funct Food 1:131–144

Lattanzio V, Morone I (1979) Variations of the orthodiphenol content in *Cynara scolymus* L. during the plant growing season. Experientia 35:993–994

Lattanzio V, Van Sumere CF (1987) Changes in phenolic compounds during the development and cold storage of artichoke. Food Chem 24(1):37–50

Llorach R., Espin JC, Tomás-Barberán FA et al (2002) Artichoke (*Cynara scolymus* L.) byproducts as a potential source of health-promoting antioxidant phenolics. J Agric Food Chem 50(12): 3458–3464

Lombardo S, Pandino G, Mauromicale G et al (2010) Influence of genotype, harvest time and plant part on polyphenolic composition of artichoke [*Cynara cardunculus* L. var. *scolymus* (L.) Fiori]. Food Chem 119:1175–1181

Lopez-Molina D, Navarro-Martınez MD, Rojas-Melgarejo F et al (2005) Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.). Phytochemistry 66:1476–1484

Meyer D, Bayarri S, Tárrega A et al (2011) Inulin as texture modifier in dairy products. Food Hydrocolloid 25:1881–1890

Nishizawa M, Fujimoto Y (1986) Isolation and structural elucidation of a new lipoxygenase inhibitor from Gardeniae fructus. Chem Pharm Bull 34:1419–1421

Nishizawa M, Izuhara R, Kaneko K et al (1987) 3-Caffeoyl-4-sinapoylquinic acid, a novel lipoxygenase inhibitor from Gardeniae fructus. Chem Pharm Bull 35:2133–2135

Nissler L, Gebhardt R, Berger S (2004) Flavonoid binding to a multi-drug-resistance transporter protein: an STD-NMR study. Anal Bioanal Chem 379:1045–1049

Pandino G, Courts FL, Lombardo S et al (2010) Caffeoylquinic Acids and Flavonoids in the Immature Inflorescence of Globe Artichoke, Wild Cardoon, and Cultivated Cardoon. J Agric Food Chem 58:1026–1031

Pandino G, Lombardo S, Mauromicale G (2013) Globe artichoke leaves and floral stems as a source of bioactive compounds. Ind Crop Prod 44:44–49

Pandino G, Lombardo S, Mauromicale G et al (2011a) Phenolic acids and flavonoids in leaf and floral stem of cultivated and wild *Cynara cardunculus* L. genotypes. Food Chem 126:417–442

Pandino G, Lombardo S, Mauromicale G et al (2011b) Profile of polyphenols and phenolic acids in bracts and receptacles of globe artichoke (*Cynara cardunculus* var. *scolymus*) germplasm. J Food Compos Anal 24:148–153

Panizzi L and Scarpati ML (1954) Constitution of Cynarine, the Active Principle of the Artichoke. Nature 174:1062–1063

Pérez-García F, Adzet T, Cañigueral S (2000) Activity of artichoke leaf extract on reactive oxygen species in human leukocytes. Free Radic Res 33(5):661–665

Pifferi PG, Vaccari A (1978) Studi sui pigmenti naturali. X. Gli antociani del carciofo (*Cynara scolymus* L.). Industr Conserv 55:107–110

Pignone D, Sonnante G (2004) Wild artichokes of south Italy: did the story begin here? Genet Resour Crop Ev 51(6):577–580

Pignone D, Sonnante G (2009) Origine ed evoluzione. In: N. Calabrese (ed) Il carciofo e il cardo. Script, Bologna, pp 2-11

Pool-Zobel B, Van Loo J, Rowland I et al (2002) Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. Brit J Nutr 87(2):273–281

Pool-Zobel BL (2005) Inulin-type fructans and reduction in colon cancer risk: Review of experimental and human data. Brit J Nutr 93(1):73–90

Portis E, Mauromicale G, Barchi L et al (2005) Population structure and genetic variation in autochthonous globe artichoke germplasm from Sicily Island. Plant Sci 168:1591–1598

Preziosi P, Loscalzo B, Marmo E (1959) Comparison of choleretic effects of CYN and Na-dehydrocholate. Experientia 15:135–138

Raccuia SA, Melilli MG, Scandurra S (2004) Potential utilisation of globe artichoke [*Cynara carduncolus* L. subsp. s*colymus* (L.) Hegi] crop residues: biomass for energy and roots for inulin production. Acta Hortic 660:607–613

Ramos PAB, Guerra ÂR, Guerreiro O et al (2013) Lipophilic Extracts of *Cynara cardunculus* L. var. *altilis* (DC): A Source of Valuable Bioactive Terpenic Compounds. J Agric Food Chem 61:8420–8429

Ramos PAB, Santos SAO, Guerra ÂR et al (2014) Phenolic composition and antioxidant activity of different morphological parts of *Cynara cardunculus* L. var. *altilis* (DC). Ind Crop Prod 61:460–471

Robenfroid MB (1999) Concepts in functional foods: The case of inulin and oligofructose. J Nutr 129:1398S–1401S

Robinson WE, Cordeiro JRM, Abdel-Malek S et al (1996) Dicaffeoylquinic Acid Inhibitors of Human Immunodeficiency Virus Integrase: Inhibition of the Core Catalytic Domain of Human Immunodeficiency Virus Integrase. Mol Pharmacol 50(4):846–855

Romani A, Pinelli P, Cantini C et al (2006) Characterization of Violetto di Toscana, a typical Italian variety of artichoke (*Cynara scolymus* L.). Food Chem 95:221–225

Rossoni G, Grande S, Galli C et al (2005) Wild artichoke prevents the age-associated loss of vasomotor function. J Agric Food Chem 53(26):10291–10296

Rottenberg A, Zohary D (1996) The wild ancestry of the cultivated artichoke. Genet Resour Crop Ev 43:53–58

Sánchez-Rabaneda F, Jáuregui O, Lamuela-Raventós RM et al (2003) Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography-tandem mass spectrometry. J Chromatogr A 1008:57–72

Sato Y, Itagaki S, Kurokawa T et al (2011) In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. Int J Pharm 403(1-2):136–138

Schauenberg P, Paris F (1977) Guide to Medicinal Plants. Keats, New Canaan.

Schütz K, Kammerer D, Carle R et al (2004) Identification and Quantification of Caffeoylquinic Acids and Flavonoids from Artichoke (*Cynara scolymus* L.) Heads, Juice, and Pomace by HPLC-DAD-ESI/MS. J Agric Food Chem 52:4090−4096

Schütz K, Persike M, Carle R et al (2006) Characterization and quantification of anthocyanins in selected artichoke (*Cynara scolymus* L.) cultivars by HPLC-DADESI-MS^n. Anal Bioanal Chem 384:1511–1517

Shimoda H, Ninomiya K, Nishida N et al (2003) Anti-Hyperlipidemic Sesquiterpenes and New Sesquiterpene Glycosides from the Leaves of Artichoke (*Cynara scolymus* L.): Structure Requirement and Mode of Action. Bioorg Med Chem Lett 13:223–228

Shukla S, Gupta S (2010) Apigenin: A Promising Molecule for Cancer Prevention. Pharm Res 27(6):962–978

Sonnante G, Carluccio AV, De Paolis A et al (2008) Identification of artichoke SSR markers: molecular variation and patterns of diversity in genetically cohesive taxa and wild allies. Genet Resour Crop Ev 55:1029–1046.

Stewart ML, Timm DA, Slavin JL (2008) Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an in vitro fermentation system. Nutr Res 28:329–334

Suchy M, Herout V, Sorm F (1960) On terpenes. CVI: on hydrogenation products of cynaropicrin, the bitter principle of artichoke (*Cynara scolymus* L.). Collect Czech Chem C 25:507−513

Takemura T, Urushisaki T, Fukuoka M et al (2012) 3,4 Dicaffeoylquinic acid, a major constituent of Brazilian propolis, increases TRAIL expression and extends the life times of mice infected with the influenza A virus. Evid-Based Compl Alt Med 946867:1−7

Tanaka YT, Tanaka K, Kojima H et al (2013) Cynaropicrin from *Cynara scolymus* L. suppresses photoaging of skin by inhibiting the transcription activity of nuclear factor-kappa B. Bioorg Med Chem Lett 23:518–523

Tárrega A, Rocafull A, Costell E (2010) Effect of blends of short and long-chain inulin on the rheological and sensory properties of prebiotic low-fat custards. Food Sci Technol 43:556–562

Van Loo J, Cummings J, Delzenne N et al (1999) Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). Brit J Nutr 81:121–132

Verpoorte R, Choi YH, Mustafa NR et al (2008) Metabolomics: Back to basics. Phytochem Rev 7:525–537

Virdis A, Motzo R, Giunta F (2009) Key phenological events in globe artichoke (*Cynara cardunculus* var. *scolymus*) development. Ann Appl Biol 155(3):419–429

Virdis A, Motzo R, Giunta F (2014) The phenology of seed-propagated globe artichoke. Ann Appl Biol 164(1):128–137

Vitor CE, Figueiredo CP, Hara DB et al (2009) Therapeutic action and underlying mechanisms of a combination of two pentacyclic triterpenes, α- and β-amyrin, in a mouse model of colitis. Brit J Pharmacol 157: 1034–1044

Wang M, Simon JE, Aviles IF et al (2003) Analysis of Antioxidative Phenolic Compounds in Artichoke (*Cynara scolymus* L.). J Agric Food Chem 51:601−608

Xiao HB, Cao X, Wang L et al (2011) 1,5-dicaffeoylquinic acid protects primary neurons from amyloid β1-42-induced apoptosis via PI3K/Akt signaling pathway. Chin Med J 124:2628–2635

Yasukawa K, Kaminaga T, Kanno H (1996) Inhibitory effect **o**f taraxastane-type triterpenes on tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. Oncology 53(4):341–344

Yasukawa K, Matsubara H, Sano Y (2010) Inhibitory effect of the flowers of artichoke (*Cynara carduncului*s) on TPA-induced inflammation and tumor promotion in two-stage carcinogenesis in mouse skin. J Nat Med 64:388–391

Zha RP, Xu W, Wang WY et al (2007) Prevention of lipopolysaccharide-induced injury by 3,5-dicaffeoylquinic acid in endothelial cells. Acta Pharmacol Sin 28(8):1143–1148

Zhu X, Zhang H, Lo R (2004) Phenolic compounds from the leaf extract of artichoke (*Cynara scolymus* L.) and their antimicrobial activities. J Agric Food Chem 52:7272–7278.

3. Metabolomic Fingerprinting of Romaneschi Globe Artichokes by NMR Spectroscopy and Multivariate Data Analysis†

3.1 Introduction

The *Cynara cardunculus* L. species is a highly polymorphic taxon of the Asteraceae family, and includes both the globe artichoke (*Cynara cardunculus* L. var. *scolymus* L. Fiori), and the cultivated cardoon (*Cynara cardunculus* L. var. *altilis* DC). Many studies support the important role of the edible parts of both globe artichoke and cultivated cardoon in human nutrition (de Falco et al., 2015). Besides, they can be considered functional foods due to their protective effects on the liver, and their anticarcinogenic, antioxidative, anticholesterol, anti-hyperlipidemic, coleretic and diuretic properties (Adzet et al., 1987; Gebhardt, 2000; Lattanzio et al., 2009; Wang et al., 2003; Shimoda et al., 2003). Globe artichoke and cardoon produce an inflorescence called capitulum or head which is partly edible and both edible and non-edible parts are a source of nutraceuticals and bio-active compounds. Some of the most characteristic ones are mono-caffeoylquinic and dicaffeoylquinic acid (Adzet and Puigmacia, 1985; Lombardo et al., 2010; Pandino et al., 2010, 2011; Schutz et al., 2004) and compounds which are not widespread such as apigenin (Pandino et al., 2013; Romani et al., 2006). The richest cultivated primary gene-pool of the globe artichoke is found in Italy, where most likely domestication occurred (Pignone and Sonnante, 2004). Classification is based on harvest time and capitulum morphological traits (Portis et al., 2005). Early varieties start producing heads in

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autumn and through spring, whereas late varieties produce heads in spring–summer only. Four groups are described based on capitulum morphology: (1) the Spinosi, bearing spines on capitulum bracts and leaves; (2) the Violetti, with purple and less spiny heads; (3) the Romaneschi, with spherical or sub-spherical non-spiny heads; (4) the Catanesi, with small, elongated and non-spiny capitulum.

Sonnante et al. (2002) and Lanteri et al. (2004) showed that morphological classifications correspond to genotypic classifications based on different genetic fingerprinting methods and principal component analysis (PCA). Nevertheless, a large genetic diversity exists within varietal groups and many local populations are found. In many cases, such populations have been collected in biodiversity lists where landraces are named after a geographical location.

A genetic analysis of local populations of Sicilian Globe artichoke within varietal groups has shown that most of the populations are genetically distinct, probably due to limited exchange of propagation materials between farmers; nevertheless, "the majority of the genetic variation is present within, rather than between populations" (Portis et al., 2005). This was ascribed by Portis et al. (2005) to different causes such as: (i) traditional methods of propagation, based on on-farm vegetative reproduction not from the best individuals, but rather from the whole field, resulting in multi-clonal populations; (ii) spontaneous mutations which are then conserved in the population due to vegetative reproduction in the absence of the meiotic sieve; (iii) reproduction from mother plants which are chimeras. While the extent of between and within-populations variability is controversial (Lanteri et al., 2001; Raccuia et al., 2004; Portis et al., 2005; Ciancolini et al., 2013), studies on the chemical composition of artichoke landraces are beginning in different areas (Lombardo et al., 2013). This is owing to the fact that the characterisation of the content of nutrients and nutraceuticals and its variability is of great interest in order to support programmes of germplasm preservation and reproduction of the best materials. Campania is one of the regions in Italy where a large number of landraces is found

and genetic characterisation has only recently begun (Rofrano et al., 2013), while studies on chemical composition are few (Dosi et al., 2013; Fratianni et al., 2007, 2014) and the comparison of landraces based on metabolic fingerprinting by NMR and chemometrics has not been performed yet.

This work aims at tracing the metabolic profile of 14 different artichoke populations within the "Romaneschi" late varietal group collected in Campania (southern Italy) and investigating the variability of the major nutraceuticals described in the artichoke. The analysis was extended to one cultivated cardoon collected in the same area. While studies on specific artichoke metabolites and the related biological activity are widespread (de Falco et al., 2015), to the best of our knowledge this is the first time that a NMR based metabolomics is used to characterise artichoke heads.

3.2 Materials and methods

3.2.1 Chemicals

First-grade dichloromethane (CH_2Cl_2) and methanol (MeOH) were purchased from Delchimica Scientific Laboratories (Naples, Italy). Deuterium oxide (99.8 atom %D) was obtained from ARMAR Chemicals (Döttingen, Switzerland) and chloroform-d (99.8 atom %D) contains 0.03% (v/v) TMS was purchased from Sigma-Aldrich (Italy).

3.2.2 Plant materials

The study was conducted on heart, internal and intermediate bracts of cultivated cardoon (*Cynara cardunculus* L.var. *altilis* DC) and globe artichoke (*Cynara cardunculus* L.var. *scolymus* L. Fiori) landraces which are traditionally grown in different areas of the Campania region in southern Italy and are classified under the "Romaneschi" late varietal group. Nine landraces were included in the study, and for two of them vegetative on-farm propagation has

resulted in different clones, which were also collected so the total population list is: Bianco di Pertosa with clones Bianco di Pertosa, zia B, zia C and zia E, Castellamare with clones Gen A, Gen B and Gen C, Capuanella, Montoro, Natalina, Pietralcina, Rosso di Paestum, Tondo Alfano, Tondo di Paestum. All populations were grown at the ex situ field for the characterisation of artichoke populations of the Campania region at CRA-ORT in Pontecagnano (southern Italy, Latitude 40°37′ N, Longitude 14°52′ E). Artichoke and cardoon heads were collected on May 2014 in triplicates and before extraction, plant materials were dried overnight under controlled temperature (60°C), powdered finely with a pestle and mortar and preserved at 4°C until use.

3.2.3 Extraction procedure

The extraction of all metabolites was made according to the procedure previously applied (Incerti et al., 2013). The dried samples (300 mg) were dissolved in 5 mL of CH_2Cl_2/MeOH/H_2O in the ratio of 2:1:1. After sonication (1 min), each mixture was centrifuged at 3000 rpm for 30 min at room temperature and then the aqueous and the organic fractions were accurately separated. The extraction was repeated twice and the solvents of each extract were pooled and evaporated to dryness under vacuum (Rotavapor R114, Büchi, Switzerland). The obtained dry residues were kept at 4°C until NMR analysis. To evaluate the reproducibility of sample preparation, three samples of each plant landrace were used to perform three replicated extractions. Samples were prepared as described earlier and analysed by NMR, and the intensity of selected signals was measured. The obtained values showed a very good repeatability, with coefficient of variation among replicates $<2.5\%$ for all signals.

3.2.4 Spectroscopic analysis

Dried aqueous fractions were diluted in 600 μL of deuterium oxide (99.8% D_2O) while dried organic fractions dissolved in 600 μL of chloroform-d (99.8% $CDCl_3$) and transferred into a 5 mm NMR tube. Dimethyl-4silapentane sodium sulfonate (DSS) (Merck, Darmstadt, Germany), added at a concentration of 0.2 mg/mL, was used as an internal standard. The NMR spectra were recorded at 298 K on a Varian Unity Inova spectrometer operating at 400 MHz. The ^1H NMR measurements were carried out with 128 transients and 16 K complex data point. The recycle time was set to 5 s, and a 45 pulse angle was used. Chemical shifts were referred to internal standard signals (δ 0.00 ppm). All spectra were processed using iNMR program (www.inmr.net), phased and baseline corrected. In total, 90 spectra (15 plant population × 2 extracted fractions × 3 replicates) were acquired. Quantification was performed by signal integration relative to the internal standard, DSS. The region of the solvent peaks was excluded from the analysis. Spectral peak assignments of all amino acids and the flavonoids, luteolin-7-rutinoside, narirutin, cyanidin-3-glucoside were obtained on the basis of pure standards, purchased by Sigma-Aldrich. Spectral peak assignments of the other detected metabolites were obtained by two-dimensional (2D) NMR experiments, and comparison with published data (de Falco et al., 2015). Metabolites were identified using pure standards and 2D ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C heteronuclear singlequantum correlation (HSQC). The ^1H connectivities were determined by 2D COSY spectra. The COSY spectra were acquired with a spectral width of 6130 Hz in both dimensions, 8 K data points, and 512 increments with 32 transients per increment. The HSQC spectra were acquired with spectral widths of 8000 Hz in the F2 dimension and 25000 Hz in the F1 dimension, a data matrix with a size of 1 K × 256 data points, and 64 transients per increment. All spectra were manually phased and baseline corrected.

3.2.5 Multivariate data analysis

Multivariate analyses were applied to ^1H NMR spectral data from both polar and organic extracts of plant materials. The ^1H NMR spectra were preliminarily normalised and reduced to integrated regions of equal widths (bins = 0.01 ppm), corresponding to 0–10 ppm and subsequently reduced to ASCII files using iNMR. Two data matrices were considered for aqueous and organic extracts, respectively, including the spectral signals recorded for each artichoke and cardoon population at each binning interval. Matrices were submitted to PCA ordination using the STATISTICA 7 Software (StatSoft Inc., Tulsa, OK, USA). In a more detailed analysis on spectral data from the polar fraction, a submatrix limited to the spectral data from the resonance region roughly corresponding to aromatic compounds ($\delta > 5.5$) was considered, and submitted to PCA.

3.3 Results and discussions

3.3.1 Metabolite profile

The ^1H NMR analysis of the aqueous and organic extracts of the studied artichoke landraces, in comparison with cardoon, showed detailed metabolite profiles. The organic extracts contained unsaturated and saturated fatty acids as the major compounds. In fact, the ^1H NMR spectra of the studied landraces (Fig. 3.1) showed signals characteristic for ω3 (δ 1.02, ω1-CH_3) and ω6 (δ 0.90, ω1-CH_3) series, in addition to saturated (δ 0.80, ω1-CH_3) fatty acids. The sesquiterpene cynaropicrin and related metabolites, reported as major components of the outer artichoke bracts of artichoke and not detected in the capitula (Ramos et al., 2013) were undetectable in the spectra. The aqueous extracts (Fig. 3.2) showed an interesting profile with the presence of metabolites belonging to different classes. Their assignment, reported in Tables 3.1–3.3, was obtained on the basis of pure standards, 2D experiments, and comparison with published data (de Falco et al., 2015). In Fig. 3.3 is reported the ^1H NMR spectrum of Bianco

di Pertosa zia E with signal assignment. Fig. 3.4 reports the chemical structure of the main detected metabolites.

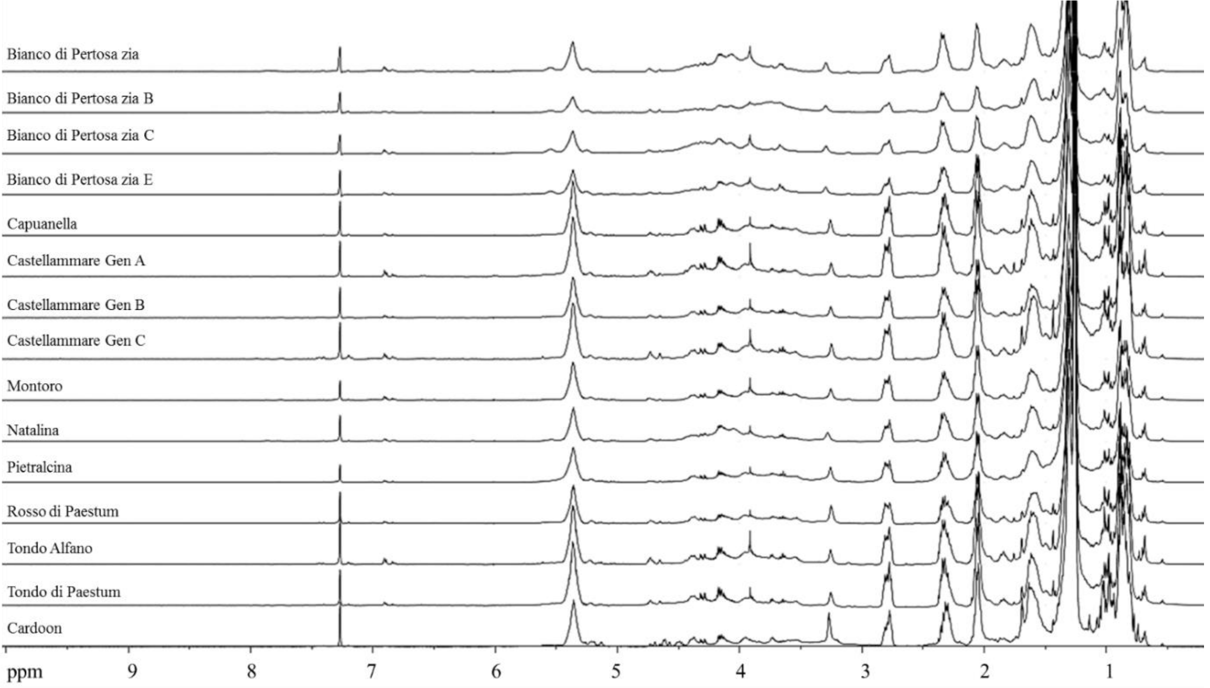

Fig. 3.1 ^1H NMR spectra in $CDCl_3$ solvent at 400 MHz of 14 globe artichokes and one cultivated cardoon. Spectral region from 0 to 10 ppm.

3.3.2 Organic acids

In the ^1H NMR spectra of the aqueous extracts of artichoke populations the following organic acids were identified by their diagnostic chemical shift value: succinic acid (SU) with characteristic singlet at δ 2.42 (α,β-CH_2), malic acid (MA) with its characteristics double doublets resonating at δ 2.39 (dd, 15.0, 10.0 Hz, β^I-CH), 2.68 (dd, 15.0, 3.0 Hz, β-CH) and 4.31 (dd, 10.0, 3.0 Hz, α-CH), formic acid (FO) with the singlet δ 8.42 (HCOOH), fumaric acid (FU) with a 2H singlet at δ 6.49 (α,β-CH = CH), citric acid (CI) with signals at δ 2.56 (d, 15.0 Hz, α,γ-CH), 2.88 (α^I,γ^I-CH). Diagnostic signals for quinic acid (QU) and shikimic acid (SH) were also detected in the ^1H NMR spectra and are reported in Fig. 3.3 and Table 3.1.

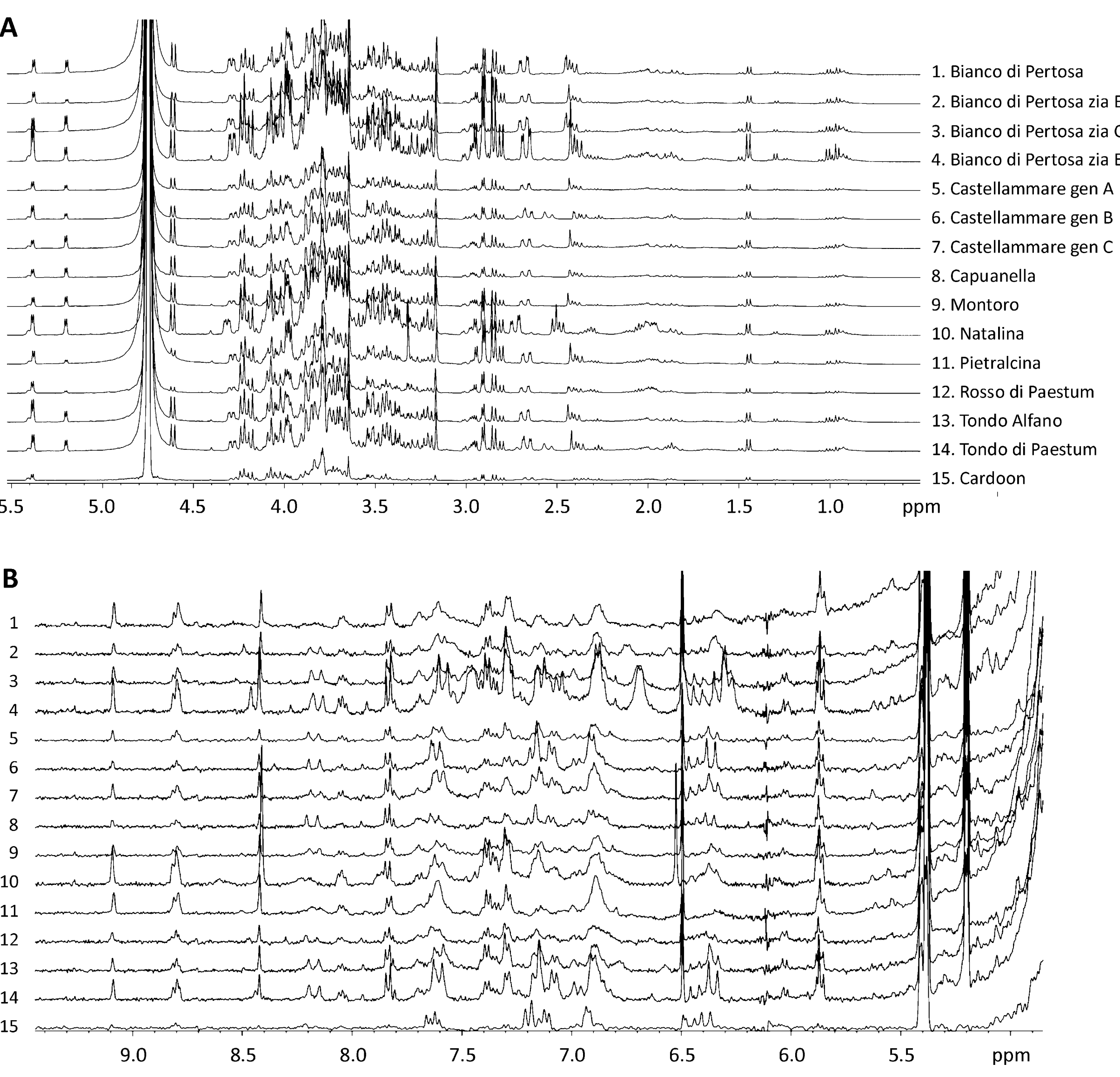

Fig.3.2 ^{1}H NMR spectra in D_2O solvent at 400 MHz of 14 globe artichokes and one cultivated cardoon: (A) spectral region from 0.5 to 5.5 ppm; (B) spectral region from 5.0 to 9.4 vertically expanded four times.

3.3.3 Free amino acids

The ^{1}H NMR spectra of artichoke aqueous extracts showed signals characteristics of the following free amino acids: isoleucine (Ile) with signals at δ 0.94 (t, 7.0 Hz, δ-CH_3) and 1.01 (d, 7.0, γ^I-CH_3), leucine (Leu) with signal at δ 0.96 (d, 7.0 Hz, δ-CH_3), threonine (Thr) with signal δ 1.29 (d, 6.0 Hz, CH_3), and alanine (Ala) with the typical methyl signals at δ 1.44 (d, 7.0 Hz, β-CH_3). Regarding lysine (Lys) and arginine (Arg), their β, γ and δ methylene groups

were also visible in the ^{1}H NMR spectra (Table 3.1). The amino acid asparagine (Asn) was easily identified in the ^{1}H NMR spectra because of its typical signals resonating each as dd at δ 2.82 (17.0, 10.0 Hz), 2.93 (17.0, 5.0 Hz) and 4.01, assigned to the methine β, $β^{I}$ and α, respectively. In addition, signals of the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) resonated in a crowded region of the spectra because of the presence of other aromatic compounds, such as polyphenols and flavonoids, present in the edible parts of artichoke at higher amounts (Table 3.1). Finally, signal attributed to glutamic acid (Glu), glutamine (Gln), and γ-aminobutyric acid (GABA) were also identified in the ^{1}H NMR spectra of the artichoke population samples (Table 3.1).

Table 3.1 ^{1}H NMR chemical shifts, assignment and multiplicity in 400 MHz spectrum of artichoke's organic acids and amino acids.

Compounds	Assignment	^{1}H (ppm)	Multiplicity [*J* (Hz)]
Organic acids			
Citric acid (CI)	α,γ-CH	2.56	d [15.0]
	α^{I},γ^{I}-CH	2.88	d [15.0]
Formic acid (FO)	HCOOH	8.42	s
Fumaric acid (FU)	α,β-CH=CH	6.49	s
Malic acid (MA)	β^{I}-CH	2.39	dd [15.0, 10.0]
	β-CH	2.68	dd [15.0, 3.0]
	α-CH	4.28	dd [10.0, 3.0]
Quinic acid (QU)	CH_2-1,1^{I}	1.88, 2.09	
	CH_2-5,5^{I}	2.0, 2.05	
	CH-3	3.55	
	CH-2	4.02	
	CH-4	4.15	
Shikimic acid (SH)	CH_2-7	2.76, 2.22	
	CH-5	3.75	
	CH-6	4.02	
	CH-4	4.43	
	CH-3	6.69	
Succinic acid (SU)	α,β-CH_2	2.42	s
Amino acids			
Alanine (Ala)	β-CH_3	1.44	d [7.0]
γ-Aminobutyric acid	β-CH_2	1.95	m
(GABA)	α-CH_2	2.30	t [7.0]
	γ-CH_2	3.01	t [7.0]
Arginine (Arg)	γ-CH_2	1.69	
	β-CH_2	1.92	
	δ-CH_2	3.33	t [7.0]
	α-CH_2	3.77	
Aspartic acid (Asp)	β-CH_2	2.71	
	α-CH	2.79	dd [3.5, 17.0]
Asparagine (Asn)	β-CH	2.82	dd [17.0, 10.0]
	β^{I}-CH	2.93	dd [17.0, 5.0]
	α-CH	4.01	[a]
Glutamic acid (Glu)	β,β^{I}-CH	2.05, 2.10	m
	γ-CH_2	2.36	m
	α-CH	3.77	
Glutamine (Gln)	β,β^{I}-CH_2	2.15	m
	γ-CH	2.47	m
	α-CH	3.63	
Isoleucine (Ile)	δ-CH_3	0.94	t [7.0]
	γ^{I}-CH_3	1.01	d [7.0]
Leucine (Leu)	δ-CH_3	0.96	d [7.0]
Lysine (Lys)	γ-CH_2	1.48	d [7.0]
	δ-CH_2	1.69	
	β-CH_2	1.88	
Phenylalanine (Phe)	CH-2,6	7.32	m
	CH-4	7.36	m
	CH-3,5	7.42	m
Threonine (Thr)	CH_3	1.29	d [6.0]
Tryptophan (Trp)	CH-10	7.19	
	CH-11	7.29	
	CH-8	7.55	
	CH-9	7.77	d [8.0]
Tyrosine (Tyr)	CH-5,9	6.90	m
	CH-6,8	7.21	m

Note: Amino acids were identified by comparison with authentic standards.
[a]Overlapped by other signals.

Table 3.2. ^{1}H NMR chemical shifts, assignment and multiplicity in 400 MHz spectrum of artichoke's carbohydrates and polyphenols.

Compounds	**Assignment**	**^{1}H (ppm)**	**Multiplicity [*J* (Hz)]**
Carbohydrates			
α-Glucose (α-Glc)	CH-4	3.25	
	CH-2	3.53	
	CH-5	3.83	
	CH-3	4.65	
	CH-1	5.21	d [4.0]
β-Glucose (β-Glc)	CH-2	3.25	
	CH-4	3.39	
	CH-5	3.46	
	CH-3	3.52	
	CH-1	4.63	d [8.0]
α-Fructofuranose (α-Fruf)	CH-5	4.05	
	CH-3	4.10	
β-Fructofuranose (β-Fruf)	CH_2-6,6^{I}	3.81, 3.65	
	CH-4	4.10	
	CH-3	4.10	
β-Fructopyranose (β-Frup)	CH_2-1,1^{I}	3.56, 3.70	
	CH-3	3.79	
	CH_2-6,6^{I}	3.81, 3.65	
	CH-4	3.88	
	CH-5	3.99	
α-Rhamnose (α-Rha)	CH_3	1.30	d [6.0]
	CH-1	5.10	d [1.0]
Inulin (Inu)	Fru-H5	3.79	[a]
	Fru-H1	3.83	[a]
	Fru-H6	3.76	[a]
	Fru-H4	4.08	[a]
	Fru-H3	4.23	[a]
	Glc-H1^{I}	5.38	d [5.0]
Caffeoyl derivatives			
3-caffeoylquinic acid	CH_2-6	1.94, 2.11	
	CH_2-2	2.15, 2.18	
	CH-4	3.64	
	CH-5	4.12	
	CH-3	5.35	
	CH-8^{I}	6.31	d [16.0]
	CH-5^{I}	6.76	
	CH-6^{I}	6.93	
	CH-2^{I}	7.03	
	CH-7^{I}	7.59	d [16.0]
5-caffeoylquinic acid	CH_2-6	2.06, 2.23	
	CH_2-2	2.04, 2.16	
	CH-4	3.72	
	CH-3	4.16	
	CH-5	5.33	
	CH-8^{I}	6.28	d [16.0]
	CH-5^{I}	6.77	
	CH-6^{I}	6.93	
	CH-2^{I}	7.04	
	CH-7^{I}	7.55	d [16.0]
1,3-dicaffeoylquinic acid Cynarin (Cyn)	CH_2-6	1.83, 2.53	
	CH_2-2	2.29, 2.88	
	CH-4	3.62	
	CH-5	4.23	
	CH-3	5.36	
	CH-8$^{I, II}$	6.32	d [16.0]
	CH-6$^{I, II}$	6.58	
	CH-2$^{I, II}$	6.81	
	CH-5$^{I, II}$	6.88	d [8.0]
	CH-7$^{I, II}$	7.58	d [16.0]
3,5-dicaffeoylquinic acid	CH_2-6	2.19, 2.23	
	CH_2-2	2.15, 2.31	
	CH-4	3.96	
	CH-5	5.37	
	CH-3	5.42	
	CH-8$^{I, II}$	6.28	d [16.0]
	CH-6$^{I, II}$	6.95	
	CH-2$^{I, II}$	7.05	
	CH-7$^{I, II}$	7.56	d [16.0]

[a]Overlapped by other signals.

Table 3.3. ^{1}H NMR chemical shifts, assignment and multiplicity in 400 MHz spectrum of artichoke's flavonoids, terpenes and other compounds.

Compounds	Assignment	^{1}H (ppm)	Multiplicity [*J* (Hz)]
Flavonoids			
Apigenin 7-rutinoside (Api)	Rha-6	1.30	d [6.5]
	CH-6	6.40	br s
	CH-8	6.44	br s
	CH-3	6.87	
	CH-3^{I}, 5^{I}	7.37	d [8.0]
	CH-2^{I}, 6^{I}	7.83	d [8.0]
Luteolin 7-rutinoside (Lut)	Rham-6	1.30	d [6.5]
	CH-6	6.40	br s
	CH-8	6.44	br s
	CH-3	6.74	
	CH-5^{I}	7.13	d [9.0]
	CH-2^{I}	7.41	br s
	CH-6^{I}	7.44	
Luteolin 7-O-β-D glucopyranoside	CH-6	6.40	br s
	CH-8	6.44	br s
	CH-3	6.74	
	CH-5^{I}	6.88	
	CH-2^{I}	7.41	br s
	CH-6^{I}	7.44	br d [8.0]
Narirutin (Nar)	Rham-6	1.07	d [6.0]
	CH-3	2.72	
	CH-2	5.52	
	CH-6, 8	6.13	
	CH-3^{I}, 5^{I}	6.79	
	CH-2^{I}, 6^{I}	7.34	d [8.0]
Cyanidin 3-O-β-glucoside (Cya)	CH-6	6.32	
	CH-8	6.68	
	CH-5^{I}	6.86	
	CH-2^{I}	7.53	
	CH-6^{I}	7.79	
	CH-4	8.46	s
Terpenes			
Cynarasaponins (Cns)	CH_3-23	0.89	s
	CH_3-24	0.92	s
	CH_3-25	0.96	s
	CH_3-26	0.99	s
	CH_3-27	1.00	s
	CH_3-28	1.04	s
	CH_2-12	5.41	
Other compounds			
adenosine (A)	Rib CH-1^{I}	6.03	d
	CH-8	8.29	s
	CH-2	8.51	
guanosine (G)	Rib CH-1^{I}	5.87	s
	CH-8	8.01	
uridine (U)	Rib CH-1^{I}	5.88	
	CH-5	7.87	
betaine (bet)	$N(CH_3)^{3+}$	3.27	s
choline (cho)	$N(CH_3)^{3+}$	3.19	s
myo-inositol (myo)	CH-4	3.27	
	CH-2,5	3.52	
	CH-3,6	3.61	
	CH-1	4.05	
Trigonelline (Tri)	CH_3	4.43	s
	CH-4	8.08	
	CH-3,5	8.80	
	CH-1	9.08	

Note: The flavonoids Api, Lut, Nar, and Cya were identified by comparison with authentic standards.

3.3.4 Carbohydrates

Different sugars were identified in the ^{1}H NMR spectra of aqueous extracts and were attributed to simple and complex saccharide units linked to different aglycones, such as the flavonoid apigenin and luteolin or in an oligosaccharide chain as in the case of inulin. The key proton signals of the main sugars found in artichoke are shown in Fig. 3.3 for Bianco di Pertosa zia E, taken as model spectrum, while the assignments are reported in Table 3.2.

Fructose and glucose were easily identified in the ^{1}H NMR spectra by means of their diagnostic anomeric proton signals (Table 3.2 and Fig. 3.3). In particular, the anomeric proton of β-D-glucose resonated at δ 4.63 as a doublet with coupling constants of 8.0 Hz, while the anomeric proton of α-D-glucose appeared at δ 5.21 as doublet with a small J coupling of 4.0 Hz. In addition, α-L-rhamnose was also identified by means of its anomeric proton signal at δ 5.10 (d, 1.0 Hz) and the typical methyl group resonating as doublet at δ 1.26 with a J value of 6 Hz. It is interesting to note in the spectrum of Bianco di Pertosa zia E (Fig. 3.3) the signals corresponding to the fructose and glucose units of inulin, resonating in the region between 3.79 and 4.23 ppm, whose intensity in the spectra is correlated with the signal at δ 5.38, thus attributed to the anomeric proton of the terminal glucose residue α-(1,2)-linked to the fructose polymer.

3.3.5 Polyphenols

Caffeoylquinic acid compounds are reported in artichoke as major metabolites (Pandino et al., 2011). Diagnostic signals of 1,3-dicaffeoylquinic acid, named cynarin (Cyn) and other mono- and dicaffeoylquinic acids were also present in the low-field region of ^{1}H NMR spectra of the population studied. In particular, signals at δ 6.28–6.32 (CH-8^{I}, CH-8^{II}) and δ 7.55–7.59 (CH-7^{I}, CH-7^{II}), both as doublets with J = 16 Hz, indicated the presence of the trans hydroxyl-cinnamoyl vinyl groups (Fig. 3.3 and Table 3.2). Moreover, the presence of a broad doublet

resonating at δ 6.88 confirmed the spin system typical of the caffeoyl moiety substituted at position 3^{I},4^{I} and 3^{II},4^{II} by hydroxyl groups.

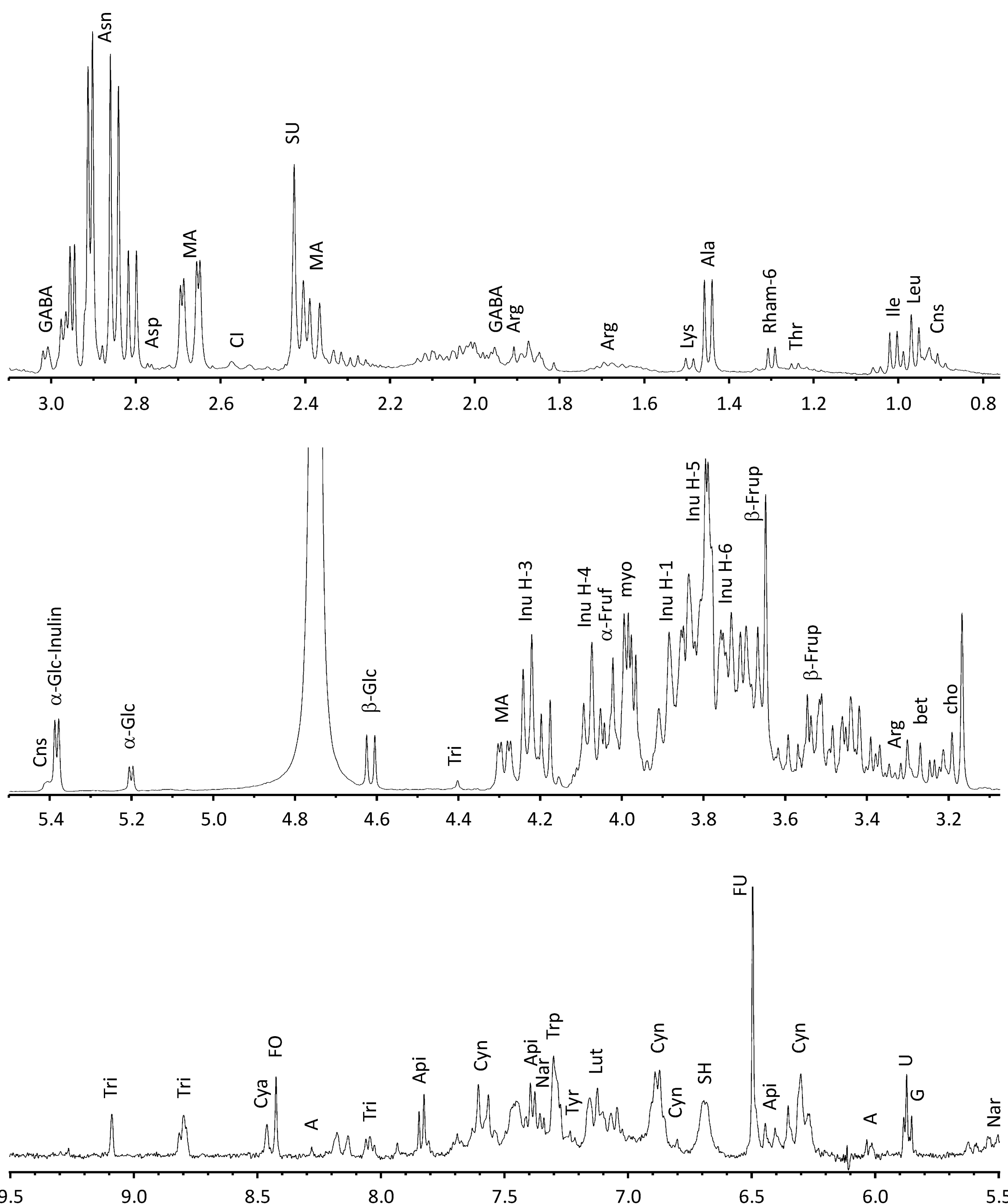

Fig. 3.3 ^{1}H NMR spectrum (D_2O, 400 MHz) of the artichoke landrace Bianco di Pertosa zia E with the identification of the compounds detected

3.3.6 Flavonoids

It is well known that the content of flavonoids is less than 10% and they are more concentrated in the leaves (Lattanzio et al., 2009). The main flavonoids found in the artichoke heads are apigenin, luteolin, naringenin and their related glycosides (Fig. 3.4). Signals for these metabolites were evidenced in the ^{1}H NMR spectra of the studied population. In particular, as reported in Fig. 3.3 and Table 3.3, key signals were found at δ 7.37 and 7.83 (each 2H, d, 8 Hz) for apigenin 7-rutinoside and at δ 7.41 (1H, bs), and 7.13 (1H, d, 9 Hz) for luteolin 7-rutinoside. Minor amounts of narirutin were also found as indicated by the signals at δ 7.34 (2H, d, 8 Hz) and 5.52 (1H), attributed to CH-2^{I},6^{I} and CH-2, respectively. In addition, the anthocyanin cyanidin 3-O-β-glucoside was also detected in the water-soluble artichoke extracts because of the characteristic singlet resonating at δ 8.46 attributed to CH-4. The remaining proton signals of the cyanidin nucleus are reported in Table 3.3.

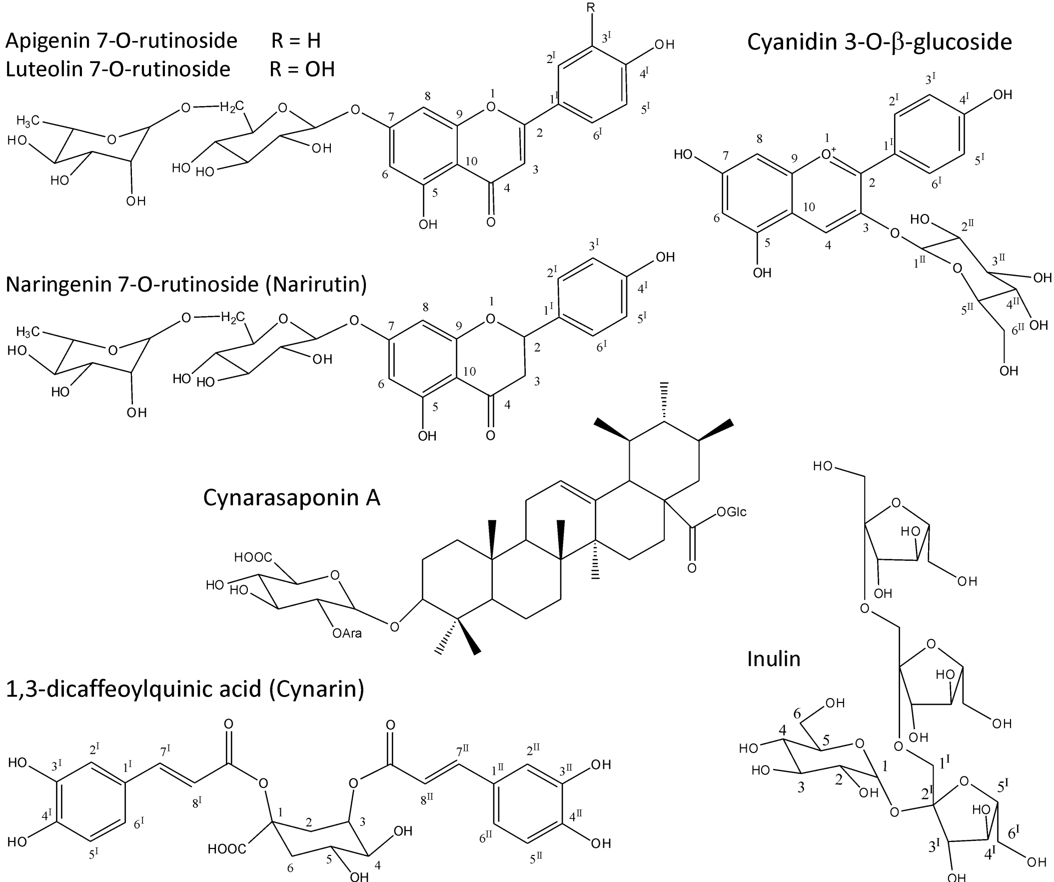

Fig. 3.4 Chemical structures of characteristic compounds found in artichoke.

3.3.7 Terpenoid glycosides

The analysis of the ^{1}H NMR spectra of the population studied showed signals characteristic of the triterpenoid saponin, cynarasaponin (Fig. 3.3). In particular, methyl signals resonated in a clear spectral zone of the spectra and therefore were easily assigned (Table 3.3).

3.3.8 PCA results

The PCA of the cardoon and artichoke landraces based on ^{1}H NMR spectral data highlighted remarkable differences among the 15 plant populations, related to their metabolomic differences. The first two components were satisfactorily explicative of the samples spectral variability, with the first two eigenvalues accounting for 86.4% (71.1 and 15.3%) and 70.4% (50.9 and 19.5%) of the total variance for the polar and apolar fractions, respectively (Fig. 3.5). In the case of apolar fraction, artichoke landraces in the PCA plot were mostly distributed along a continuous gradient, with the exception of Bianco di Pertosa zia B and Pietralcina, plotted at the top and bottom of the bidimensional space defined by the first two principal components, respectively (Fig. 3.5). For the other landraces, limited to apolar compounds, such pattern indicates a general similarity of metabolomic fingerprinting among all samples. Indeed, the main differences were determined by only three spectral signals, resonating at δ 0.9, δ 1.3 and δ 1.4. The first signal, diagnostic of ω1-CH_3 of the ω6 series, was positively correlated to the second PCA axis, consistent with the highest and lowest content of these fatty acids in Bianco di Pertosa zia B and Pietralcina landraces, respectively, compared to the other samples. Differently, the signal resonating at δ 1.3, related to saturated alkyl chain of fatty acids, was negatively correlated to the first PCA axis, indicating a high content in most of the landraces, and a lower content in Bianco di Pertosa zia B and Pietralcina, which showed positive loadings on that axis. Finally, the signal resonating at δ 1.4, negatively related to both PCA axes, although not easily associable to types of compounds, indicated protons eventually more

abundant in Pietralcina landraces, as well as in all samples plotted in the left bottom quadrant of the PCA space.

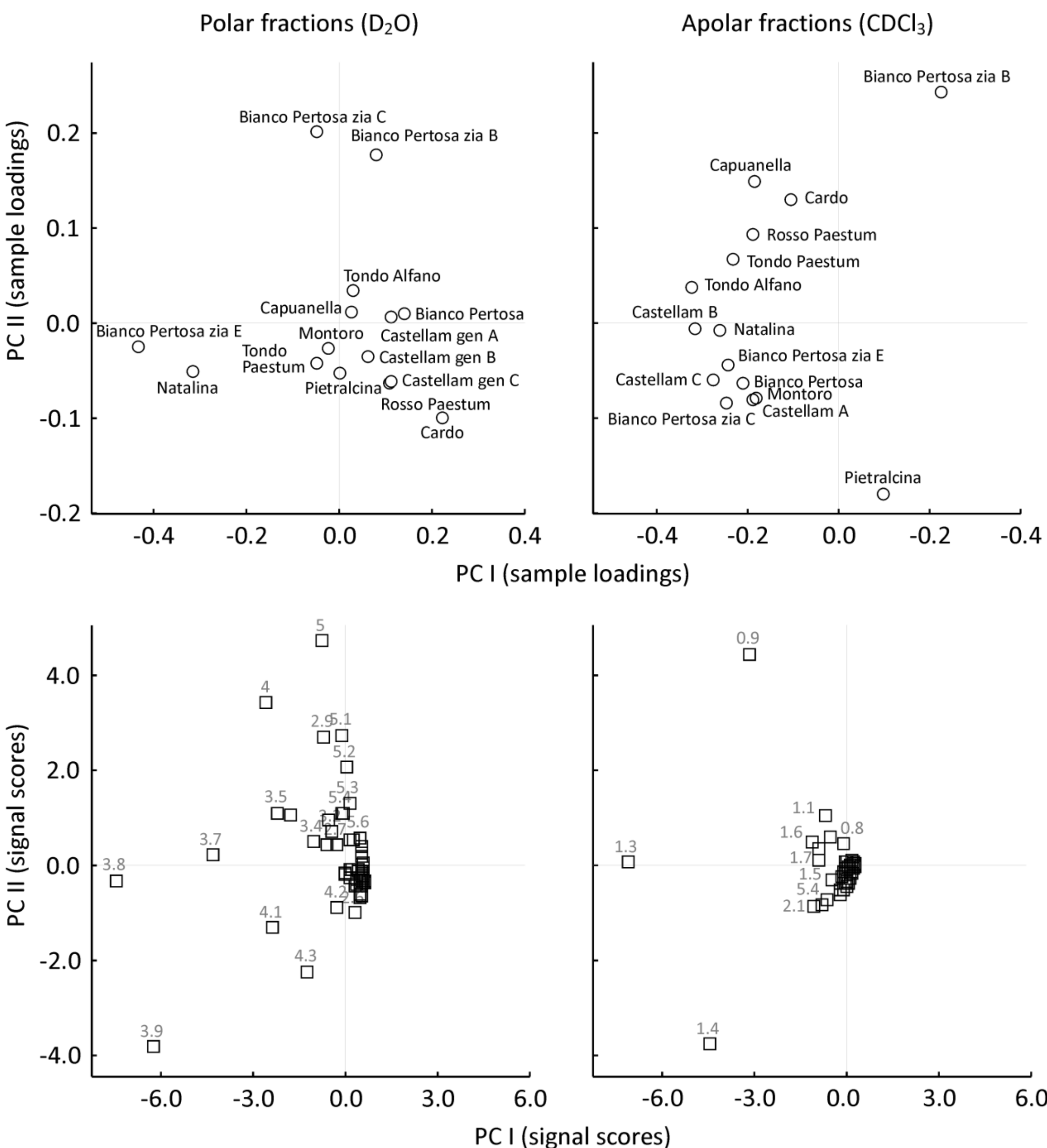

Fig. 3.5 Principal component analysis (PCA) ordination of 14 artichoke landraces and one cardoon based on ^{1}H NMR resonance spectra from aqueous (left, top and bottom) and organic (right, top and bottom) fractions. Top: variable loadings; bottom: signal scores.

The bidimensional PCA plot of the ^{1}H NMR spectral data from the aqueous fraction (Fig. 3.5) clearly separated the samples based on two main gradients, corresponding to increasing content of compounds resonating at δ 3.7–4.3 and δ 5.0–5.3, pointing to the bottom-left and top in the bidimensional space defined by the first two principal components, respectively. The first spectral region includes signals corresponding to H-1, H-3, H-4, H-5 and H-6 of inulin, most abundant in Bianco di Pertosa zia E and Natalina (Table 3.4). The signals resonating at δ 5.0–5.3, corresponding to other glycosides, are most abundant in all Bianco di Pertosa artichokes, with the exception of zia E. Conversely, cardoon, showing the lowest content of inulin and other glycosides, was found at the bottom rightmost location in the principal component space (Fig. 3.5). The artichoke landraces plotted near the principal component space origin were hardly separated, showing a more homogeneous distribution of spectral signals corresponding to aromatic compounds (Fig. 3.5).

Table 3.4 Relative abundance (in mg/mL of the sample tube) of major metabolites identified by ^{1}H-NMR analysis in aqueous extracts of artichoke cultivars and cardoon, as calculated from ^{1}H NMR peaks intensity

Plant cultivar	Inu	other glycosides	Api, Lut	Cyn	A, G, U	Total (mg/mL)
Bianco di Pertosa	4.271±0.195	0.921±0.016	0.025±0.001	0.025±0.001	0.085±0.001	5.327±0.128
Bianco di Pertosa zia B	4.125±0.006	1.145±0.020	0.111±0.002	0.171±0.008	0.015±0.001	5.567±0.167
Bianco di Pertosa zia C	8.394±0.095	1.534±0.067	0.211±0.007	0.242±0.007	0.034±0.001	10.415±0.046
Bianco di Pertosa zia E	16.231±0.337	0.445±0.008	0.111±0.005	0.131±0.006	0.175±0.005	17.093±0.305
Castellam gen A	4.537±0.046	0.654±0.025	0.016±0.001	0.043±0.001	0.067±0.001	5.317±0.010
Castellam gen B	5.411±0.034	0.591±0.022	0.051±0.002	0.061±0.001	0.015±0.001	6.129±0.009
Castellam gen C	3.936±0.034	0.334±0.009	0.021±0.001	0.016±0.001	0.012±0.001	4.319±0.183
Capuanella	7.711±0.050	0.715±0.019	0.045±0.002	0.055±0.001	0.081±0.001	8.607±0.174
Montoro	11.434±0.024	0.881±0.039	0.081±0.001	0.074±0.003	0.067±0.003	12.537±0.272
Natalina	18.667±0.493	0.686±0.002	0.134±0.006	0.106±0.002	0.221±0.008	19.814±0.258
Pietralcina	6.845±0.055	0.855±0.037	0.031±0.001	0.015±0.001	0.035±0.002	7.781±0.184
Rosso di Paestum	4.752±0.201	0.297±0.011	0.022±0.001	0.018±0.001	0.018±0.001	5.107±0.033
Tondo Alfano	7.985±0.249	0.781±0.002	0.061±0.001	0.071±0.002	0.085±0.003	8.983±0.252
Tondo di Paestum	7.271±0.312	0.525±0.025	0.061±0.002	0.075±0.001	0.045±0.001	7.977±0.340
Cardoon	0.911±0.040	0.054±0.001	0.004±0.001	0.008±0.001	0.001±0.001	0.978±0.040

Note: For each metabolite, peaks reported in Tables 3.1–3.3 were considered. Cultivars are ranked as in Fig. 3.1 to facilitate a comparison between tabulated data and ^{1}H NMR spectra. Data refers to mean and standard deviation of three replicated spectra for each cultivar.

Indeed, such signals, resonating at δ higher than 5.5, were all grouped in a dense cloud close to the centre of the principal component space (Fig. 3.5). The PCA of the samples based on the ^{1}H NMR spectral data from the aromatics resonance region extended the previous findings, showing metabolomic differences among the artichoke populations related to different abundance of specific aromatic compounds (Fig. 3.6).

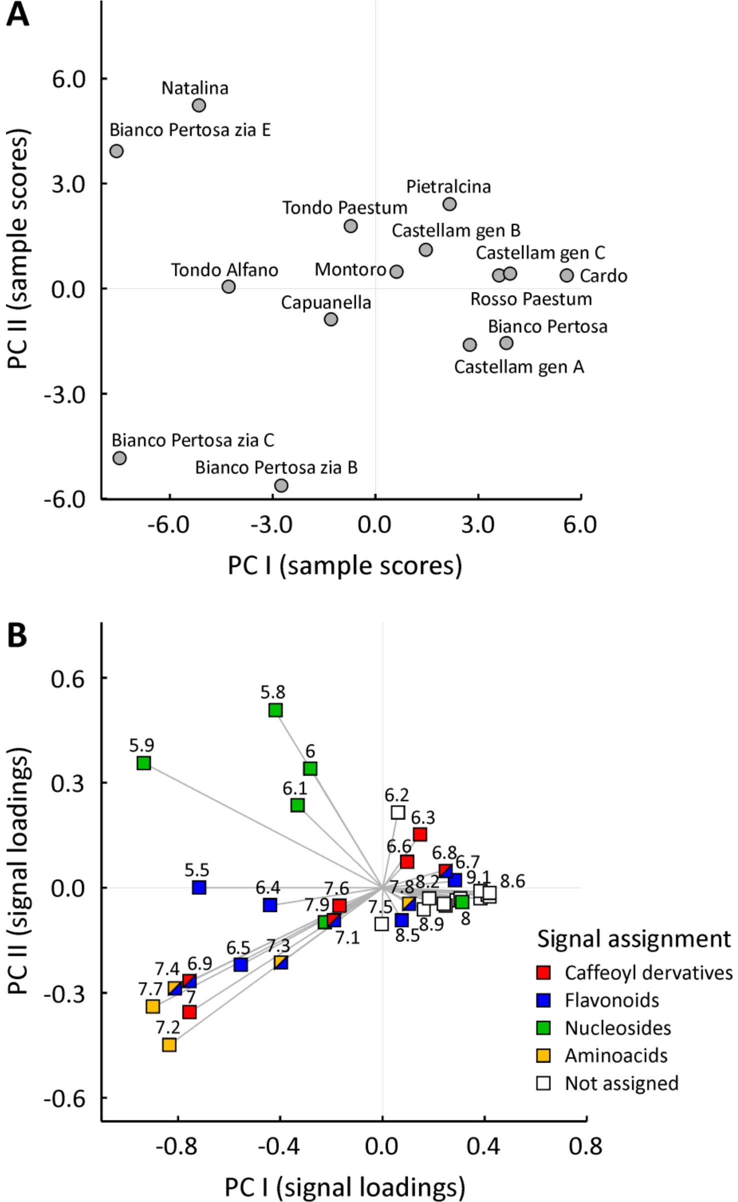

Fig. 3.6 Principal component analysis (PCA) ordination of ^{1}H NMR resonance intervals from δ 5.7 to δ 10.0, roughly corresponding to aromatic compounds, based on values recorded in artichoke landraces and cardoon: (A) sample scores; (B) loading vectors. Resonance intervals are coloured according to ^{1}H NMR signal assignment and labelled by centre of parts per metre range. Explained variance was 41.9% and 21.3% for principal component I and II (PCI and PCII), respectively.

Considering the flavonoids apigenin and luteolin, as well as the cynarin 1,3-dicaffeoylquinic acid, their diagnostic signals (see Table 3.3) were distributed in the bottom-left direction of the principal component space, extending along a gradient of increasing content of such compounds in the artichoke samples (please compare the spectral peaks intensity in Table 3.4 with the sample scores in Fig. 3.6). Indeed, such compounds showed only some slight differences of relative abundance in the tested artichoke populations, being most abundant in the Bianco di Pertosa Zia B, Zia C, and Zia E landraces, relatively abundant in the Natalina, Tondo Alfano, and Tondo di Paestum populations, and rather rare in the Rosso di Paestum and Pietralcina artichokes, as well as in the cardoon (Table 3.2). Interestingly, a restricted interval of signals resonating at δ 5.8-6.1, possibly associated with nitrogen bases of nucleic acids (i.e. adenosine, guanosine, uridine, Table 3.1) were distributed along the top-left direction in the principal component space, indicating a gradient of increasing abundance of the corresponding compounds in the tested plant samples. Such signals showed important differences of relative abundance in the artichoke samples compared to flavonoids and caffeoylquinic acids, being mostly abundant in Bianco di Pertosa Zia E and Natalina, and lowest in cardoon (Table 3.4).

3.4 Conclusion

Metabolomic approach coupling NMR spectroscopy with multivariate data analysis allowed for a detailed metabolite profile of the tested cardoon and artichoke populations to be provided. Considering the taxonomic relatedness of the studied plant materials, we observed relevant differences in relative content of several types of organic compounds, including organic acids, amino acids, carbohydrates, caffeoyl derivatives, flavonoids, sesquiterpenes, triterpenes, and nucleosides. Our findings are generally consistent with previous studies on metabolites identification in artichoke heads (de Falco et al., 2015) that reported inulin and caffeoyl derivatives as the main bioactive metabolites. However, few studies specifically reported on artichoke metabolic profiling at landrace and population level. Among these, the study by Dosi

et al. (2013) reported a detailed nutritional profile obtained by classical methods of the "Capuanella" landrace, also included in our analysis. In particular, Dosi et al. (2013) reported n-6 linoleic and palmitic acids as the most abundant fatty acid (72% of the total fatty acids), and lower content of ascorbic (13.70 mg/100 g), and folic acid (65.00 µg/ 100 g). Phenolic compounds, mainly the chlorogenic acid (425.46 mg/100 g) were relatively abundant. Leucine (Leu) and lysine (Lys) were the most abundant essential amino acids while Asx (aspartic acid plus asparagine) and Glx (glutamic acid plus glutamine) were the most representative non-essential amino acids. A direct quantitative comparison with our results is not to be recommended, due to methodological differences related to both plant growth conditions and analytical techniques and tools (Lattanzio and Morone, 1979; Lattanzio and Van Sumere, 1987; Lombardo et al., 2010). However, all the metabolites detected by Dosi et al. (2013) were here recognised by NMR spectroscopy and in comparable amounts. In particular, as general examples, the amino acid asparagine (Asn) was easily identified in the ^{1}H NMR spectra because of the typical signals of the methine β, β^{I} and α, with a high intensity of such signals reflecting the high amount of this amino acid in the artichoke heads (Dosi et al., 2013) and in other food plants (Lea et al., 2007).

The majority of phytochemistry studies reporting on *Cynara scolymus* investigated specific classes of compounds (de Falco et al., 2015). Among these, the works by Pandino et al. (2010) and Pinelli et al. (2007) are of particularly interest reporting on edible parts of different globe artichokes, focusing on caffeoylquinic acids and flavonoids. In particular, Pandino et al. (2010), analysed "Tondo di Paestum" together with two Sicilian globe artichoke varieties and wild and cultivated cardoon, showing the highest content of luteolin glucoside among the studied samples, as well as moderate content of apigenin glucuronide and caffeoylquinic acids. Caution should be posed in comparing such findings with our results, due to relevant methodological differences between the studies and the difference in the plant materials. However, the observations by Pandino et al. (2010) are consistent with ours (Table 3.4) for "Tondo di

Paestum" that in our study has intermediate values of polyphenols among the 14 different set of studied populations and high value, as found by Pandino et al. (2010) compared to cardoon. In a pharmacological analysis of antimicrobial and quorum quenching activity of the extract of the Montoro artichoke, an ecotype also included in our analysis, Fratianni et al. (2014) found a good content of polyphenols, among which a high amount of chlorogenic acid and cynarin was detected by ultra-pressure liquid chromatography (UPLC). Our results are consistent with such findings, with characteristic ^{1}H NMR spectral peaks of these polyphenols (Table 3.1) being recognisable in the spectra of our plant materials. In our data, variation in metabolites is not only found among landraces but also between different clones collected within the Bianco di Pertosa and Castellammare landraces. This is consistent with results of Lombardo et al. (2013) who report large differences in polyphenol content and antioxidant activity between clones of two Sicilian artichoke landraces, and suggest to use this information for the selection of elite clones and to choose the appropriate clone for different uses (e.g. fresh consumption or food processing). The obtained results represent a wider metabolic fingerprinting and can therefore provide the basis for improving the selection of reproductive materials. Within the Bianco di Pertosa landrace, for instance, clone Zia E has shown the highest content of nutraceuticals and can therefore be proposed as an interesting material for vegetative reproduction. While no similar studies are found on metabolic fingerprinting among and within artichoke landraces, the general finding that a large variability exists among clones within artichoke landraces is supported by the genetic studies of Portis et al. (2005), where differences within landraces are shown to be large, and this is attributed to farmers' methods of vegetative reproduction, resulting in multi-clonal populations.

Under a general perspective, our results showed that the combined approach of ^{1}H NMR spectroscopy and multivariate data analysis can provide very detailed metabolomic profiles of biological samples. As an example, previous studies showed that, among the minor compounds in the plant, terpenoid glycosides are found in artichoke, generally based on sesquiterpenoid

and triterpenoid aglycon (Ramos et al., 2013). Our findings of signal characteristics in the artichoke heads of the triterpenoid saponin cynarasaponin are consistent with such observations, showing that our approach can be used not only to assess major compounds in biological samples, but also to detect small fractions of specific, minor molecules. From a methodological point of view, previous metabolomic studies on artichoke were mostly based on advanced ultra-high performance liquid chromatography (UHPLC) techniques, both coupled (Farag et al., 2013; El Senousy et al., 2014) or uncoupled (Abu-Reidah et al., 2013) with multivariate statistics. The use of ^{1}H NMR as an analytical technique and multivariate data analysis as an exploratory statistical tool have been widely used to characterise plant metabolomics (e.g. Verpoorte et al., 2008; Incerti et al., 2013), and recently applied to Jerusalem artichoke (*Helianthus tuberosus* L.) to assess metabolic changes in overwintering tubers of three plant varieties (Clausen et al., 2012). In this respect, our work is the first application coupling ^{1}H NMR with multivariate statistics to provide a metabolomic fingerprinting of *Cynara scolymus*.

References

Abu-Reidah IM, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A. 2013. Extensive characterization of bioactive phenolic constituents from globe artichoke (*Cynara scolymus* L.) by HPLC-DAD-ESI-QTOF-MS. *Food Chem* 141: 2269–2277.

Adzet T, Camarasa J, Laguna JC. 1987. Hepatoprotective activity of polyphenolic compounds in isolated rat hepatocytes from *Cynara scolymus* against CCl_4 toxicity: in isolated rat hepatocytes. *J Nat Prod* 50: 612–617.

Adzet T, Puigmacia M. 1985. High-performance liquid chromatography of caffeoylquinic acid derivatives of *Cynara scolymus* L. leaves. *J Chromatogr A* 348: 447–453.

Ciancolini A, Alignan M, Pagnotta MA, Vilarem G, Crinò P. 2013. Selection of Italian cardoon genotypes as industrial crop for biomass and polyphenol production. *Ind Crops Prod* 51: 145–151.

Clausen M R, Bach V, Edelenbos M, Bertram HC. 2012. Metabolomics reveals drastic compositional changes during overwintering of Jerusalem artichoke (*Helianthus tuberosus* L.) tubers. *J Agric Food Chem* 60: 9495-9501.

de Falco B, Incerti G, Amato M, Lanzotti V. 2015. Artichoke: botanical, agronomical, phytochemical, and pharmacological overview. *Phytochem Rev* 14: 993-1018.

Dosi R, Daniele A, Guida V, Ferrara L, Severino V, Di Maro A. 2013. Nutritional and metabolic profiling of the globe artichoke (*Cynara scolymus* L. 'Capuanella' heads) in province of Caserta, Italy. *Austr J Crop Sci* 7: 1927-1934.

El Senousy AS, Farag MA, Al Mahdy DA, Wessjohann LA. 2014. Developmental changes in leaf phenolics composition from three artichoke cvs. (*Cynara scolymus*) as determined via UHPLC-MS and chemometrics. *Phytochemistry* 108, 67-76.

Farag MA, El-Ahmady SH, Elian FS Wessjohann LA. 2013. Metabolomics driven analysis of artichoke leaf and its commercial products via UHPLC-q-TOF-MS and chemometrics. *Phytochemistry* 95: 177-187.

Fratianni F, Pepe R, Nazzaro F. 2014. Polyphenol composition, antioxidant, antimicrobial and quorum quenching activity of the "Carciofo di Montoro" (*Cynara cardunculus* var. scolymus) Global artichoke of the Campania region, southern Italy. *Food Nutr Sci* 5: 2053-2062.

Fratianni F, Tucci M, De Palma M, Pepe R, Nazzaro F. 2007. Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynara cardunculus* L. var. scolymus (L.) Fiori). *Food Chem* 104: 1282-1286.

Gebhardt, R. 2000. Choleretic and anticholestatic activities of flavonoids of artichoke (*Cynara carduncul*us L. subsp. scolymus L. Hayek). *Acta Hortic* 681: 429–436.

Incerti G, Romano A, Termolino P. Lanzotti V. 2013. Metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis as a tool for biodiversity informatics: a case study on the classification of *Rosa x damascena*. *Plant Biosys* 147: 947–954.

Lanteri S, Di Leo I, Ledda L, Mameli MG, Portis E. 2001. RAPD variation within and among populations of globe artichoke cultivar ''Spinoso sardo''. *Plant Breed* 120: 243–246.

Lanteri S, Saba E, Cadinu M, Mallica GM, Baghino L, Portis E. 2004. Amplified fragment length polymorphism for genetic diversity assessment in globe artichoke. *Theor Appl Gen* 108: 1534–1544.

Lattanzio V, Kroon PA, Linsalata V, Cardinali A. 2009. Globe artichoke: A functional food and source of nutraceutical ingredients. *J Func Foods* 1: 131-144.

Lattanzio V, Morone I. 1979. Variations of the orthodiphenol content in *Cynara scolymus* L. during the plant growing season. *Experientia* 35: 993–994.

Lattanzio V, Van Sumere CF. 1987. Changes in phenolic compounds during the development and cold storage of artichoke. *Food Chem* 24: 37–50.

Lea PJ, Sodek L, Parry MAJ, Shewry PR, Halford NG. 2007. Asparagine in plants. *Ann Appl Biol* 150: 1–26.

Lombardo S, Pandino G, Mauromicale G. 2013. Total polyphenol content and antioxidant activity among clones of two Sicilian globe artichoke landraces. *Acta Hortic* 983: 95-101.

Lombardo S, Pandino G, Mauromicale G, Knödler M, Carle R, Schieber M. 2010. Influence of genotype, harvest time and plant part on polyphenolic composition of globe artichoke [*scolymus* (L.) Fiori]. *Food Chem* 119: 1175-1181.

Pandino G, Courts F, Lombardo S, Mauromicale G, Williamson G. 2010. Caffeoylquinic acids and flavonoids in the immature inflorescence of globe artichoke, wild cardoon, and cultivated cardoon. *J Agric Food Chem* 58:1026-1031.

Pandino G, Lombardo S, Mauromicale G., Williamson G. 2011. Phenolic acids and flavonoids in leaf and floral stem of cultivated and wild *Cynara cardunculus* L. genotypes. *Food Chem* 126: 417-422.

Pandino G, Lombardo S, Williamson G, Mauromicale G. 2013. Flavonoids content of *Cynara cardunculus* L. wild and cultivated germplasm accessions. *Acta Hortic* 983: 81-86.

Pignone D, Sonnante G. 2004. Wild artichokes of south Italy: did the story begin here? *Gen Res Crop Evol* 51: 577-580.

Pinelli P, Agostini F, Comino C, Lanteri S, Portis E, Romani A. 2007. Simultaneous quantification of caffeoyl esters and flavonoids in wild and cultivated cardoon leaves. *Food Chem* 105: 1695–1701.

Portis E, Mauromicale G, Barchi L, Mauro R, Lanteri S. 2005. Population structure and genetic variation in autochthonous Globe artichoke germplasm from Sicily Island. *Plant Sci* 168: 1591-1598.

Raccuia SA, Mainolfi A, Mandolino G, Melilli MG. 2004. Genetic diversity in *Cynara carduncculus* revealed by AFLP markers: comparison between cultivars and wild types from Sicily. *Plant Breed* 123: 280–284.

Ramos PAB, Guerra ÂR, Guerreiro O, Freire CSR, Silva AMS, Duarte MF, Silvestre AJD. 2013. Lipophilic Extracts of *Cynara cardunculus* L. var. *altilis* (DC): a source of valuable bioactive terpenic compounds. *J Agric Food Chem* 61: 8420–8429.

Rofrano G, Trotta N, Pepe R, Pagnotta MA, Saccardo F, Cardi T. 2013. Improving the artichoke quality of Campania region. *Acta Hortic* 983: 31-37.

Romani A, Pinelli P, Cantini C, Cimato A, Heimler D. 2006. Characterization of Violetto di Toscana, a typical Italian variety of artichoke (*Cynara scolymus* L.). *Food Chem* 95: 221–225.

Schutz K, Kammerer D, Carle R, Schieber A. 2004. Identification and quantification of caffeoylquinic acids and flavonoids from artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC-DAD-ESI/MSn. *J Agric Food Chem* 52: 4090-4096.

Shimoda H, Ninomiya K, Nishida N, Yoshino T, Morikawa T, Matsuda H, Yoshikawa M. 2003. Anti-hyperlipidemic sesquiterpenes and new sesquiterpene glycosides from the leaves of artichoke (*Cynara scolymus* L.): structure requirement and mode of action. *Bioorg Med Chem Lett* 13: 223–228.

Sonnante G, De Paolis A, Lattanzio V, Perrino P. 2002. Genetic variation in wild and cultivated artichoke revealed by RAPD markers, *Gen Res Crop Evol* 49: 247–252.

Verpoorte R, Choi YH, Mustafa NR, Kim HK 2008. Metabolomics: Back to basics. *Phytochem Rev* 7: 525–537.

Wang M, Simon JE, Aviles IF, He K, Zheng QY, Tadmor Y. 2003. Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). *J Agric Food Chem* 51: 601−608.

4. Chia seeds products: an overview[‡]

4.1 Introduction

Salvia hispanica L. (Lamiaceae), also known as chia, is an annual herbaceous plant, native of southern Mexico and northern Guatemala. The genus *Salvia* consists of ca 900 species (Ayerza, 2005) and its name comes from the latin word “salvere”, referring to the curative properties of the well known culinary and medicinal herb *Salvia officinalis* (Dweck, 2005). Nowadays, some species are still used all over the world for their nutritional properties and their beneficial effect on human health. The species *S. hispanica* produces numerous dry indehiscent fruits which are commonly called seeds. These small white and dark seeds in pre-Columbian times, along with corn, beans and amaranth, were one of the basic foods in the diet of several Central American civilizations including Mayan and Aztec populations. The seeds had also been used like a tribute to the capital of Aztec Empire (Codex Mendoza 1542) and offered to Aztec gods (de Sahagun 1579). Due to its religious implications, chia was banned under the rule of the European conquerors and was re-discovered in the 1990s. since then it has spread in Argentina, Australia, Bolivia, Colombia, Guatemala, Mexico and Peru and outside America, in Australia, Africa and Europe (Bochicchio et al. 2015). Chia is a macrothermal short-day flowering plant. This means that chia needs to be sown in late spring and will not flower until late summer or fall at high latitudes; therefore, its chances of producing seed are low since grain filling is hampered by frost (Ayerza and Coates 2005). As there was no source of natural long day chia available, Jamboonsri et al. 2012 developed early flowering chia germplasm by genetic mutations. The metabolomic profile of four chia seeds early flowering genotype, G3, G8, G17,

[‡] de Falco, B., Amato, M., & Lanzotti, V. (2017). Chia seeds products: an overview. Manuscript submitted

W13.1, was studied by de Falco et al. (2016) and compared to the profile of commercial black and white seeds by ^{1}H NMR spectroscopy coupled with multivariate data analysis. Results showed that commercial black seeds have highest content of carbohydrates, while commercial white and long-day flowering genotypes showed a lower content of these metabolites. The relative content of the identified amino acids was significantly lowest in the G3 and highest in G17 which also showed the highest content of saturated and unsaturated fatty acids. Chia seeds commercialized today have a coat color ranges from black and black spotted to white. Ayerza 2013a showed that there is no difference in the chemical composition between two genotypes Tzotzol and Iztac, which produce black-spotted and white seeds, respectively. Chia seeds is also used to increase the ω–3 fatty acid content of animal products like eggs, poultry and rabbit (Peiretti et al. 2008). Several classes of secondary metabolites belong to the sage seeds such as flavonoids and their glycosides, polyphenols, which are mainly composed by caffeic acid building block, anthocyanins and proanthocyanidins. Fiber is one important component of chia seeds studied for its insoluble and soluble fraction. It can be used as foam stabiliser, suspending agent and emulsifier for food and pharmaceutical purpose due to its physical properties (Reyes-Caudillo et al. 2008) including water holding capacity and viscosity (Vázquez-Ovando et al. 2009). However, the chemical composition and the amount of each class of compounds in chia seeds vary depending on several factors including genetic modifications, environmental conditions and agricultural practices.

4.2 Chemical constituent

Chia seeds have a very important role as functional food and nutritional supplement (Coelho et al. 2014). The composition and the concentration of their bioactive compounds depend on several factors: climatic conditions, geographical origin and by the extraction methods (Ayerza & Coates, 2004, 2009a, b, 2011; Capitani et al. 2012; Ixtaina et al. 2011). Seeds are composed

by total dietary fiber from 47.1 to 59.8% (Weber et al. 1991) and contain up to 40% of oil with high content of unsaturated fatty acids, of which α-linolenic acid represents up to 68% (Ayerza 1995; Taga et al. 1984). Moreover, they are a good source of proteins (12 - 26%), dietary fiber, vitamins, minerals and antioxidants (Bushway et al. 1981). These data capture the attention of researchers because in the last few years there was an increasing interest in all of these compounds (Capitani et al. 2012; Ayerza and Coates 2004, 2011). Furthermore, chia seeds do not contain toxic compounds and gluten, thus making seeds a safe ingredient also for gluten free diets (Menga et al. 2017).

4.3 Caffeic acid derivatives

Caffeic acid plays an important role from both chemical and biological point of view in chia seeds extract. This phenolic acid, composed by a dihydroxy-phenyl group linked with acrylic acid, represents the molecular skeleton of several metabolites in the Lamiaceae family. Caffeic acid, also classified as hydroxycinnamic acid, can be bound to quinic acid in different positions to give rise to a class of metabolites named caffeoylquinic acids, of which chlorogenic acid is the most abundant in the polar extract of chia seeds (Martínez-Cruz et al. 2014). Moreover, in the metabolome of chia seeds, are presents monomers of caffeic acid building block but also condensation products such as polymers (Table 4.1). Monomeric derivatives, including caffeic acid itself and ferulic acid, have been isolated from chia seeds (Ixtlahuacán, Colima, Mexico) by ultra-high performance liquid chromatography (UHPLC) (Martínez-Cruz et al. 2014). The authors found a concentration of caffeic acid (0.0274 mg/g) higher than that reported for mango (0.0077 mg/g), papaya (0.0159 mg/g) and blueberry (0.0216 mg/g), but lower than that reported for peach (0.0371 mg/g) (Balasundram et al., 2006). Ayerza 2013a, after HPLC analysis, also reported the chlorogenic acid as the most abundant phenol (0.222 mg/g) followed by caffeic acid (0.144 mg/g). These results are in agreement with those reported by Reyes-Caudillo et al.

2008, who also analyzed chia seeds from Mexico using by HPLC. Particularly, he found chlorogenic acid as the most abundant phenols followed by caffeic acid, but the concentrations are slightly lower (0.102 and 0.003 mg/g respectively) if compared to Ayerza results. On the contrary, Coelho et al. 2014 showed a high content of caffeic acid among phenols. Caffeic acid dimers are also frequent in chia samples and among them rosmarinic acid is the most abundant one. Martínez-Cruz et al. 2014 also reported the rosmarinic acid as the major phenolic compound of chia seeds (0.9267 mg/g). Several biological activities have been described for rosmarinic acid such as antioxidant, astringent, anti-inflammatory, antithrombotic, antimutagen, antibacterial and antiviral (Huang and Zhang, 1991; Parnham and Kesselring, 1985; Zou et al. 1992). Trimers and tetramers of caffeic acid building block, including salvianolic acid A-K and lithospermic acid, were reported from other Salvia species such as *S. miltiorrhiza*, *S. officinalis, S. cavaleriei*, *S. flava*, *S. chinensis* (Ai et al. 1994; Ai and Li 1992; Lo and Foo 1999 and 2001; Zhang and Li 1994). However, from the best of our knowledge, there are no reports showing the presence of salvianolic and lithospermic acids in chia seeds.

4.4 Flavonoids

Flavonoids, ubiquitous compounds present in plants, belong to a polyphenolic subclass having a fifteen-carbon skeleton which consist of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C). They are the major responsible for color, taste and prevention of fat oxidation in food (Yao et al., 2004). Flavonoids have many biochemical activities such as antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer and antiviral (Critchfield et al. 1996; Cushnie et al. 2005; Li et al. 2000; Zandi et al. 2011, Zhu et al. 2012). They are widely distributed in chia seeds and their synthesis increase as a result of microbial infection (Dixon et al. 1983). Taga et al. 1984 reported the presence of myrcetin, quercetin and kaempferol in methanolic hydrolyzed extracts of chia seeds and evaluated their antioxidant activity (see below

and Table 4.1). Reyes-Caudillo et al. (2008) also studied both hydrolyzed and crude extracts of chia seeds obtained from two different regions of Mexico. They identified quercitin-phenolic glycosides and kaempferol-phenolic glycosides as the major components of the crude extract. After hydrolysis of the extract the authors quantified the free aglycon forms as quercitin 0.150 mg/g and 0.268 mg/g and kaempferol 0.360 mg/g e 0.509 mg/g in Jalisco and Sinaloa seeds, respectively. On the contrary, Ayerza 2013a reported myrcetin as the major flavonols in the Tzotzol and Iztac chia seeds genotypes (0.115 and 0.121 mg/g respectively) followed by kaempferol and quercitin. Another research on chia seeds var. Chionacalyx from Mexico was achieved by Martínez-Cruz et al., 2014, who detected daidzin, glycitin, genistin, glycitein, and genistein as the major isoflavones in the phenolic extract. Daidzin was found at the concentration of 0.066 mg/g of sample. To note that recently Lowe et al., 2008 reported such compound at high concentration (4.685 mg/g) in Kudzu roots, *Pueraria lobate*, as naturally occurring anti-alcohol-addiction agent in complex with human mitochondrial aldehyde dehydrogenase.

Table 4.1 Caffeic acids derivatives and flavonoids from *Salvia hispanica* seeds

Chemical constituent	Quantification	Origin	Analytical technique	Reference
Caffeic acids derivatives				
Caffeic acid	0.0274 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014
	0.139 - 0.149 mg/g	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	0.003 – 0.006 mg/g	Jalisco and Sinaloa (Mexico)	HPLC	Reyes-Caudillo et al. 2008
	0.030 mg/g	l.m. (São Paulo, Brazil)	UPLC	Coelho et al. 2014
	6.6 x 10^{-3} mol/kg	l.m. (West Lafayette, US)	TLC, GLC and UV	Taga et al. 1984
Ferulic acid	T	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014
Chlorogenic acid	0.226 – 0.218 mg/g	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	0.102 – 0.045 mg/g	Jalisco and Sinaloa (Mexico)	HPLC	Reyes-Caudillo et al. 2008
	0.004 mg/g	l.m. (São Paulo, Brazil)	UPLC	Coelho et al. 2014
Rosmarinic acid	0.9267 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014

Flavonoids				
Myricetin	3,1 x 10^{-3} mol/kg	l.m. (West Lafayette, US)	TLC, GLC and UV	Taga et al. 1984
	0.115 – 0.121 mg/g	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
Quercetin	0.2 x 10^{-3} mol/kg	l.m. (West Lafayette, US)	TLC, GLC and UV	Taga et al. 1984
	0.150 – 0.268 mg/g	Jalisco and Sinaloa (Mexico)	HPLC	Reyes-Caudillo et al. 2008
	0.007 – 0.006 mg/g	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	0.17 µg/g	l.m. (São Paulo, Brazil)	UPLC	Coelho et al. 2014
Kaempferol	1.1 x 10^{-3} mol/kg	l.m. (West Lafayette, US)	TLC, GLC and UV	Taga et al. 1984
	0.360 – 0.509 mg/g	Jalisco and Sinaloa (Mexico)	HPLC	Reyes-Caudillo et al. 2008
	0.025 – 0.024 mg/g	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
Daidzin	0.006 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014
Glycitein	0.0005 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014

Glycitin	0.0014 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014
Genistein	0.0051 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014
Genistin	0.0034 mg/g	Chionacalyx (Mexico)	UHPLC	Martínez-Cruz et al. 2014

Note: T = traces, l.m. = purchased from local market

4.5 Oil composition

Since ancient times oil extracted from chia seeds has been used in traditional medicine against eye infections and for the treatment of stomach disorders (Lu and Foo 2002; Reyes-Caudillo et al. 2008). The oil content of the seeds ranges from 25% to 50% and contains high concentrations of polyunsaturated fatty acids (Bushway et al. 1981; Taga et al. 1984) (Table 4.2 and Fig. 4.1). Research demonstrated that oil extracted from chia seeds also contain several phenolic compounds such as tocopherols, phytosterols and carotenoids with their related antioxidant activity that play a very important role in the deterioration of the oil due to lipid oxidation (Matthaus 2002; Ixtaina et al. 2011). It was widely demonstrated that in *S. hispanica* seeds ω-3 is the most abundant component among fatty acids, in particular, the content of α-linolenic acid (C18:3) is over than 50% of all fatty acids (Palma et al. 1947; Ayerza 1995, 2011; Segura-Campos et al. 2014). Therefore, chia seed can be considered as a natural source of ω-3 which

play a very important role in human nutrition and in human health due to its anti-inflammatory, antiarrhythmic and antithrombotic activity (Garg et al., 2006; Geelen et al. 2004; Din et al. 2004, Wall et al. 2010). Da Silva Marineli et al. 2014 characterized the chia seed oil from Chile using the positive ion easy ambient sonic-spray ionization mass spectrometry (EASI MS) technique and reported ranks of fatty acids abundance in the following order: α-linolenic acid (62.8%), linoleic acid (18.23%), palmitic acid (7.07%), oleic acid (7.04%) and stearic acid (3.36%). These results are in agreement with those reported in other studies (Ayerza, 1995; Ayerza & Coates, 2004). Amato et al. (2015) reported the first data on the quality of chia seeds produced in Europe, from an experiment conducted in Basilicata (South Italy), particularly the oil extracted from Italian chia seeds was not significantly different from those grown in traditional area (Peru) and in a new area (Australia). However, the oil extracted in Italy was more rich in chlorophyll, carotenoids and α-linolenic acid but showed a higher free acidity and peroxides. As mentioned previously, chemical composition and oil yield can be affected by several factors such as extraction technique and geographical area. For example, Ixtaina el al. 2011 used two extraction techniques to obtain oil from chia seeds purchased from different source, Argentina and Guatemala. In both seeds, the oil yield was much lower in pressing than in solvent extraction (20.30% and 24.8% compared to 26.70% and 33.6%, respectively). Another important example is the study conducted on the effect of six different ecosystems of South America on the protein and oil contents, fatty acid composition and peroxide index of chia seeds from Argentina (Ayerza and Coates 2004; Ayerza 2013b). The authors demonstrated that the chemical composition of the seeds is widely affected by the location and environmental factors such as temperature, light and soil type.

Table 4.2 Chemical constituents from Oil of chia seeds

Chemical constituent	Quantification %	Origin	Analytical technique	Reference
Polyunsaturated fatty acids				
Arachidonic acid (C20:4)	0.13	I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
Eicosatrienoic acid (C20:3)	0.01	I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	0.03	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
α-linolenic acid (C18:3)	62.02	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	64.5 and 63.3	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	57.71 and 58.39	Peru and Australia	HPLC-MS	Amato et al. 2015
	68.52	I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	69.0	I.m. (West Lafayette, US)	GLC	Taga et al. 1984
	62.80	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	63.4 62.7 62.4 52.0 60.7	Catamarca (Argentina)	GLC	Ayerza 1995
	63.20 57.50 58.55 54.20 62.00 62.20 61.65 64.20	Northwestern Argentina	GLC	Ayerza & Coates, 2004
	64.5 and 66.7 65.6 and 69.3	Argentina and Guatemala	Pressing and solvent extract, GC	Ixtaina el al. 2011
Linoleic acid (C18:2)	17.5 and 18.4	Tzotzol and Iztac	HPLC	Ayerza 2013b
	18.82 and 20.74	Peru and Australia	HPLC-MS	Amato et al. 2015
	17.36	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014

	15.3	I.m. (West Lafayette, US)	GLC	Taga et al. 1984
	20.40	I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	18.23	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	19.8 20.2 20.8 20.3 20.3	Catamarca (Argentina)	GLC	Ayerza 1995
	18.00 19.25 19.10 20.50 20.30 20.10 21.05 18.35	Northwestern Argentina	GLC	Ayerza & Coates, 2004
	20.3 and 17.5 19.7 and 16.6	Argentina and Guatemala	Pressing and solvent extract, GC	Ixtaina el al. 2011
Monounsaturated fatty acids Oleic acid (C18:1)	6.65 and 6.8	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	10.55	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	7.30 and 7.04	Peru and Australia	HPLC-MS	Amato et al. 2015
	7.6	I.m. (West Lafayette, US)	GLC	Taga et al. 1984
	2.43	I.m. (I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	7.04	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	7.3 7.8 7.3 7.6 8.2	Catamarca (Argentina)	GLC	Ayerza 1995
	3.40 3.50 10.30 13.25 7.15 6.75 6.85 6.90	Northwestern Argentina	GLC	Ayerza & Coates, 2004

	5.4 and 5.5 5.3 and 5.8	Argentina and Guatemala	Pressing and solvent extract, GC	Ixtaina el al. 2011
Palmitoleic acid (C16:1)	0.09	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	T	I.m. (West Lafayette, US)	GLC	Taga et al. 1984
	0.06	I.m. (I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	0.08	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
Saturated fatty acids Stearic acid (C18:0)	2.67	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	3.65 and 4.1	Tzotzol and Iztac (Ecuador)	HPLC	Ayerza 2013b
	2.99 and 3.19	Peru and Australia	HPLC-MS	Amato et al. 2015
	2.9	I.m. (West Lafayette, US)	GLC	Taga et al. 1984
	0.29	I.m. (I.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	3.36	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	3.3 3.1 3.1 3.1 3.7	Catamarca (Argentina)	GLC	Ayerza 1995
	3.40 3.50 3.55 3.55 2.95 2.75 2.75 3.00	Northwestern Argentina	GLC	Ayerza & Coates, 2004
	3.1 and 4.4 3.0 and 2.7	Argentina and Guatemala	Pressing and solvent extract, GC	Ixtaina el al. 2011
Margaric acid (C17:0)	0.06	I.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	0.07	I.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014

Palmitic acid (C16:0)	12.32 and 10.17	Peru and Australia	HPLC-MS	Amato et al. 2015
	5.2	l.m. (West Lafayette, US)	GLC	Taga et al. 1984
	6.5 and 6.2	Tzotzol and Iztac (Ecuador)	GC	Ayerza 2013b
	6.69	l.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
	7.47	l.m. (Yucatan, Mexico)	GC-MS	Segura-Campos et al. 2014
	7.07	l.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	6.2 6.3 6.4 7.1 6.9	Catamarca (Argentina)	GLC	Ayerza 1995
	7.25 7.65 7.60 7.65 6.55 7.40 6.95 7.15	Northwestern Argentina	GLC	Ayerza & Coates, 2004
	6.6 and 5.9 6.2 and 5.5	Argentina and Guatemala	Pressing and solvent extract, GC	Ixtaina el al. 2011
Pentadecanoic acid (C15:0)	0.05	l.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	0.03	l.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
Myristic acid (C14:0)	0.07	l.m. (Santiago, Chile)	GC-EASI(+)-MS	da Silva Marineli et al. 2014
	0.03	l.m. (São Paulo, Brazil)	GC	Coelho et al. 2014
Tocopherols				
α-Tocopherol	7.53 - 7.46 mg/kg	Peru and Australia	HPLC	Amato et al. 2015
δ-Tocopherol	12.99 - 13.45 mg/kg	Peru and Australia	HPLC	Amato et al. 2015
γ-Tocopherol	457.38 - 489.52 mg/kg	Peru and Australia	HPLC	Amato et al. 2015
	225 and 325 mg/kg 250 and 410 mg/kg	Argentina and Guatemala	Pressing and solvent extract, HPLC	Ixtaina el al. 2011
Pigment				
Chlorophyll	1.80 - 2.40 mg/kg	Peru and Australia	spectrophotometry	Amato et al. 2015

Note: % of total fatty acids; mg/kg of oil chia seed; T = trace; l.m. = purchased from local market

Fig. 4.1 Chemical structures of fatty acids from chia seeds

4.6 Fibers

Chia seeds constitute a potential ingredient in food industry applications due to its dietary fiber content. Since the early 1950s, it was discovered the importance of the fibers for human health and nutrition. On 1953, Hipsley first coined the multiple term “dietary fiber”. Later on, Trowell redefined the term as the remnants of plant components that are resistant to hydrolysis by human alimentary enzymes (Hipsley 1953, Trowell 1976). Nowadays the definition is broader including not only the plant components but all the carbohydrate polymers with ≥10 monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans (Codex Alimentarius Commission, 2009). Dietary fiber is a class of compounds including oligosaccharides and polysaccharides such as cellulose and hemicellulose that may be associated with other components (e.g., lignin, pectins, gums and mucilage). The total dietary fiber (TDF) has become an important component of the diet, especially for their physiological functionality based on the swelling property after water absorption, due to the presence of carbohydrates with free polar groups that interact with hydrophilic links within the matrix leading to formation of gel and consequent increase of peristalsis. Published reports indicate

that many health benefits are associated to the intake of TDF. In fact, the fiber has prebiotic effect and it is active on coronary heart disease, stroke, hypertension, diabetes, obesity and gastrointestinal disorders (Lairon et al. 2005; Liu et al. 1999; Montonen et al. 2003; Petruzziello et al. 2006; Steffen et al. 2003; Whelton et al. 2005). Chia seed is a good source of TDF, which are composed by soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). Particularly, the SDF are partially expelled from the seed as mucilaginous gel when it comes in contact with water, and fermented in the colon. On the contrary, IDF may only be fermented to a limited extent in the colon (Anderson et al. 2009). TDF in chia seeds from Chile were analysed using enzymatic gravimetric AOAC method by da Silva Marineli et al. 2014 which reported higher amount of TDF (37.50 g/100g) with predominant IDF (35.07 g/100g), these findings agree with other reports (Capitani et al. 2012; Craig and Sons 2004; Weber et al. 1991), but lower amount were reported by Ayerza 2013a (TDF 24.56 g/100g with IDF 14.35 g/100g). The same analytical technique was used by Reyes-Caudillo et al. 2008, who characterized TDF in Jalisco and Sinaloa seeds (*S. hispanica* L.), particularly, the SDF and IDF content of Jalisco seeds were 6.84 and 34.9 g/100g, respectively, while in Sinaloa seeds 6.16 and 32.87 g/100g, respectively. The main component found in IDF was the Klason lignin, which plays an important role in the protection of unsaturated fats and it is responsible for the hypocholesterolemic activity associated with fiber intake (Tolba et. 2011). The percentage of neutral sugars was also reported in both fractions, 13.79–14.97% and 4.69-5.12% for IDF and SDF, respectively. Highest amount of fiber-rich fraction (FRF) was also detected in *S. hispanica* seeds from Mexico by Vázquez-Ovando et al. 2009. Particularly they evaluated the FRF obtained by dry processing of defatted flour of chia seeds and reported 29.56 g/100g of crude fiber content and 56.46 g/100g of TDF content, of which 53.45 g/100g was IDF. Compared to other reports, these values clearly show that dry fractionation with 100 mesh effectively concentrated TDF content. A part of the fiber in chia is located in the outer cells of the fruit and is partly extruded from the fruit surface upon hydration in the form of a clear mucilaginous capsule which adheres firmly

to the fruit itself. Capitani et al. 2013 described this process using scanning electron microscopy (SEM) after 5, 10, 30 and 60 min after chia seeds become wetted. Chia mucilage is part of the SDF (Ayerza and Coates 2001; Reyes-Caudillo et al. 2008) and in order to obtain high amount of mucilage, Muñoz et al. (2012a) performed the extraction with different seeds/distilled water ratio, pH and temperature condition. An optimum yield value (7%) was achieved at 80°C with pH = 8 and seed/water ratio of 1:40. Mucilage is mainly composed by sugars as xylose, glucose and glucuronic acid, but little is known about whole chemical structure. From the best of our knowledge, the only tentative structural identification was proposed by Lin et al. 1994, who obtained β-D-xylose, α-D-glucose and 4-O-methyl-α-D-glucuronic acids by acid hydrolysis and characterized a tetrasaccharide with 4-O- methyl -α-D-glucoronopyranosyl residues occurring as branches of β-D-xylopyranosyl on the main chain by using mass spectrometry and ^{13}C NMR spectroscopy.

4.7 Total polyphenolic content and their antioxidant activity

Chia seeds and oil contain a large number of natural antioxidant such as tocopherols, phytosterols, carotenoids (Álvarez-Chávez et al. 2008), polyphenolic compounds which are mainly constructed from the caffeic acid building block and flavonoids, including the flavones myricetin, quercetin and kaempferol. This class of compounds is the main responsible for the antioxidant activity of chia seeds due to their ability to scavenge free-radicals, to chelate metal ions and to donate hydrogens. In particular, the B ring of flavones is the major responsible of ROS and RNS scavenging activity because the transfer of a hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, that stabilize them giving rise to a relatively stable flavonoid radical (Cao et al. 1997). Antioxidant compounds reduce the risk of chronic diseases including cancer and heart disease, they offer protection against some disorders such atherosclerosis, stroke, diabetes and neurodegenerative diseases such as Alzheimer and

Parkinson (Vuksan et al. 2007; Wu et al. 1998; Yagi et al. 1989; Zhao et al. 1996). The highest amount of total polyphenol was found by Martínez-Cruz et al. 2014 (1.6398 ± 0.2081 mgGAE/g of chia seed, *S. hispanica* L var. Chionacalyx) who developed an ultrahigh performance liquid chromatography (UHPLC) method for the analysis of phenolic compounds and isoflavones content. Although, the results of Amato et al., 2015 are lower (0.53-0.98 mgGAE/g of chia seed), they are in agreement with other studies (de Falco et al. 2017; da Silva Marineli et al. 2014; Porras-Loaiza et al. 2014; Reyes-Caudillo et al. 2008; Coelho et al. 2014). The antioxidant activity of hydrolysed and nonhydrolyzed extract of chia seeds was also evaluated by using the oxidation reaction of β-carotene and linoleic acid (Miller 1971). Results showed flavanols glycosides as the major antioxidant in the nonhydrolyzed extract followed by chlorogenic acid and caffeic acid, while in the hydrolysed fraction caffeic acid is the major antioxidant source and myricetin has ca. 1.5 times the activity of quercetin followed by kaempferol (Taga et al. 1984). Other methods were used over the years to evaluate the antioxidant activity, for example $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and FRAP were used by Sargi et al. 2013 to analyse chia seeds obtained from Brazil and they reported 2.56 ± 0.03; 1.72 ± 0.09 and 2.86 ± 0.10 mmol TEAC/g, respectively. Antioxidant activity, quantified with the $ABTS^{\bullet+}$ decolorization assay, was also evaluated on chia seeds obtained from Mexico and Argentina, but lower amount was detected, 0.446 and 0.488 mmol TEAC/g, respectively (Capitani el al. 2012; Vázquez-Ovando et al. 2009).

4.8 Industrial uses

Dietary fibers in chia seeds have not only physiological functionality for their beneficial effect on human health but also technological functionality which greatly depends on hydration properties (Borderìas et al. 2005). These are water-holding and absorption capacity, solubility and swelling, viscosity and gelling. Gum can be extracted from dietary fiber fraction of chia

seeds for use as an additive to control viscosity, stability, texture, and consistency in food systems. Segura-Campos et al. 2014 highlighted important physicochemical properties of chia gum for the food industry such as a very good ability to water holding (110.5 g/g). They also compared functional properties of fatted and defatted chia gum reporting lower oil holding ability (11.67 g/g) and water absorption (36.26 g/g) in defatted gum, and greater retention oil holding (25.79 g/g) and water absorption (44.08 g/g) in fatted gum. Vazquez-Ovando et al. 2009 obtained FRF from defatted chia flour to determine its possible applications in products requiring hydration. The FRF water-holding capacity was 15.41 g/g, higher than reported for soy bean, wheat and maize hulls (Mongeau & Brassard, 1982; Yeh et al. 2005). This may be due to the particular structure of the mucilage and to hemicellulose and lignin ratio. In contrast, chia FRF had a low oil-holding capacity of 2.02 g oil/g sample. They also evaluated other two important properties of chia FRF, that were the emulsifying activity, which is the ability to facilitates the solubilization or dispersion of two immiscible liquids, and the emulsifying stability, the ability to maintain an emulsion (53.26 mL/100 mL and 94.84 mL/100 mL, respectively). Its emulsifying activity may be due to the high content of protein 28.14 g/100g in FRF, which are strong emulsifying agents (Pearce et al. 1978). It can be therefore a valid alternative in foods as foam stabilizer and emulsifier. Microstructural features of chia seeds were also studied by light and scanning electron microscopy by Muñoz et al. 2012a, who explained the great capacity of chia mucilage hydration reporting a water retention of 27 times of its own weight, almost double that those reported by Vázquez-Ovando et al. (2009), in which only the FRF was hydrated. Later on, they produced a mixture of mucilage of *S. hispanica* and whey protein concentrates in proportions 1:3 and 1:4 as a new source of polymer blends to develop coatings and edible films which may be used as protective water vapor barrier (Muñoz et al., 2012b). It is also used as such or in whole-seeds as a component of biodegradable film (Capitani et al. 2016), thickening agent for bread and pasta, especially in gluten-free

formulations (e.g. Menga et al. 2017), and for anti-corrosion (Hermoso-Diaz et al. 2014), cosmetic use and medical uses (Vuksan et al. 2010).

4.9 Conclusion

Salvia hispanica L. is a plant known since ancient times whose seeds were used as a basic food in the diet of Mayan and Aztec populations. Chia seeds are a good source of nutraceuticals and a number of reports have shown their beneficial effects on human health due to their chemical composition. They are rich in dietary fiber and polyunsaturated fatty acids, especially α-linolenic acid. *S. hispanica* seeds also contain high amount of polyphenols, including caffeic and chlorogenic acids, myricetin, quercetin and kaempferol, which give rise to high antioxidant activity. Due to its mucilaginous gel, chia seeds can be also used in cosmetic, pharmaceutical and food companies as protective agent against moisture, foam stabilizer and emulsifier agents for its particular composition rich in carbohydrates. However, further studies are needed to fully clarify the molecular structure of chia mucilage.

References

Ai CB, Deng QH, Song WZ, Li LN (1994) Salvianolic acid J, a depside from *Salvia flava*. Phytochemistry 37:907–908

Ai CB, Li L-N. (1992) Salvianolic acids D and E: Two new depsides from *Salvia miltiorrhiza*. Planta Med 58:197–199

Álvarez-Chávez LM, Valdivia-López MDLA, Aburto-Juarez MDL, Tecante A (2008) Chemical characterization of the lipid fraction of Mexican Chia seed (*Salvia hispanica* L.). Int J Food Prop 11: 687-697

Amato M, Caruso MC, Guzzo F, Commisso M, Bochicchio R, Galgano F, Labella R, Favati F (2015) Nutritional quality of seeds and leaf metabolites of Chia (*Salvia hispanica* L.) from Southern Italy. Eur. Food Res & Tech, Vol. 241, Issue 5, p615

Anderson JW, Baird P, Davis RH, Ferreri S, Knudtson M, Koraym A, Williams C L (2009) Health benefits of dietary fiber. Nutr Rev 67:188–205

Ayerza R (1995) Oil content and fatty acid composition of Chia (*Salvia hispanica* L.), from five northeastern locations in northwestern Argentina. J Am Oil Chem Soc 72:1079–1081

Ayerza R (2005) Chia: Rediscovering a Forgotten Crop of the Aztecs. The University of Arizona. Press. Tucson, Arizona, USA.

Ayerza R (2013a) Seed composition of two Chia (*Salvia hispanica* L.) genotypes which differ in seed color Emir. J Food Agric 25: 495–500

Ayerza R (2013b) Composition of Chia (*Salvia hispanica*) grown in six tropical and subtropical ecosystems of South America. J. Food Agric. 25:495–500

Ayerza R and Coates W (2001) Chia seeds: new source of omega-3 fatty acids, natural antioxidants and dietetic fiber Southwest Center for Natural Products Research and Commercialization, Office of Arid Lands Studies, Tucson, AZ

Ayerza R Coates W (2004) Composition of chia (*Salvia hispanica* L.) grown in six tropical and subtropical ecosystems of South America. Trop Sci 44:131–135

Ayerza R, Coates W (2005) The renaissance of Chia. In: Chia: rediscovering a forgotten crop of the Aztecs. The University of Arizona Press, Tucson

Ayerza R, Coates W (2009a) Some quality components of four Chia (*Salvia hispanica* L.) genotypes grown under tropical coastal desert ecosystem conditions. Asian J Plant Sci 8:301–307

Ayerza R, Coates W (2009b) Influence of environment on growing period and yield, protein, oil and linolenic content of three Chia (*Salvia hispanica* L.) selections. Ind Crop Prod 30:321–324

Ayerza R, Coates W (2011) Protein content, oil content and fatty acid profiles as potential criteria to determine the origin of commercially grown Chia (*Salvia hispanica* L.). Ind Crop Prod 34:1366–1371

Balasundram N, Sundram K, Samman S. (2006) Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence and potential uses. Food chem 99:191–203

Bochicchio R, Philips TD, Lovelli S, Labella R, Galgano F, Di Marsico A, Perniola M, Amato M. (2015) Innovative Crop Productions for Healthy Food: The Case of Chia (*Salvia hispanica* L.) In: A. Vastola (ed.) The Sustainability of Agro-Food and Natural Resource Systems in the Mediterranean Basin 2015, Springer-Verlag Berlin and Heidelberg pp 15-27.

Borderìas AJ, Sanchez-Alonso I, Pérez-Mateos M. (2005) New applications of fibres in food : Addition to fishery products. Trends in Food Sci Technol 16:458–465

Bushway AA, Belyea PR, Bushway RJ (1981) Chia seed as a source of oil, polysaccharide, and protein. J Food Sci 46:1349–1350

Cahill JP (2003) Ethnobotany of Chia, *Salvia hispanica* L. (Lamiaceae). Econ Bot 57:604–618

Cao G, Sofic E, Prior RL (1997) Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Rad Biol Med 22:749–760

Capitani MI, Ixtaina VY, Nolasco SM, Tomás M.C. (2013) Microstructure, chemical composition and mucilage exudation of Chia (*Salvia hispanica* L.) nutlets from Argentina. J Sci Food Agric 93:3856–3862

Capitani MI, Matus-Basto A, Ruiz-Ruiz JC, Santiago-García JL, Betancur-Ancona DA, Nolasco SM, Tomás MC, Segura-Campos MR (2016) Characterization of Biodegradable Films Based on *Salvia hispanica* L. Protein and Mucilage. Food Bioprocess Technol 9:1276 1286 DOI 10.1007/s11947-016-1717-y

Capitani MI, Spotorno V, Nolasco SN, Tomas MC (2012) Physicochemical and functional characterization of by-products from Chia (*Salvia hispanica* L.) seeds of Argentina. LWT— Food Sci Technol 45:94–102

Codex Alimentarius Commission; Food and Agriculture Organization; World Health Organization. Report of the 30th session of the Codex Committee on nutrition and foods for special dietary uses. ALINORM 09/32/26. 2009

Codex Mendoza (1542) Edition of Francisco del Paso and Troncoso (1925). Museo Nacional de Arqueologia, Historia y Etnografia, Mexico

Coelho SM, de las Mercedes Salas-Mellado M (2014) Chemical characterization of Chia (*Salvia hispanica* L.) for use in food product. J Food Nutr Res 2:263–269

Craig R, Sons M (2004) Application for approval of whole Chia (*Salvia hispanica* L.) seed and ground whole Chia as novel food ingredients. Advisory committee for novel foods and processes (pp. 1–29). Ireland: Company David Armstrong

Critchfield JW, Butera ST, Folks TM. (1996) Inhibition of HIV activation in latently infected cells by flavonoid compounds, AIDS Res. and Human Retroviruses, vol. 12, no. 1, pp. 39–46

Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Agents 26: 343–356

da Silva Marineli R, Moraes ÉA, Lenquiste SA, Godoy AT, Eberlin MN, Maróstica M. R. (2014) Chemical characterization and antioxidant potential of Chilean Chia seeds and oil (*Salvia hispanica* L.) Food Science and Technology 59 1304-1310

de Falco B, Incerti G, Bochicchio R, Phillips TD, Amato M, Lanzotti V (2017) Metabolomic analysis of *Salvia hispanica* seeds using NMR spectroscopy and multivariate data analysis. Ind Crop Prod 99: 86-96

de Sahagun B (1579) Historia general de las cosas de Nueva España. Reprinted 1982 by School of American Research, Santa Fe, NM

Din JN, Newby DE, Flapan AD (2004) Omega 3 fatty acids and cardiovascular disease—fishing for a natural treatment. Brit Med J 328:30–5

Dixon RA, Dey PM, Lamb CJ (1983) Phytoalexins: enzymology and molecular biology. Adv Enzymol R A M B 55:1–136

Dweck, A. C. The folklore and cosmetic use of various Salvia species. In Sage: The Genus Salvia; Kintzios, S. E., Ed.; Harwood Academic Publishers: Amsterdam, The Netherlands, 2005; pp 1−25.

Garg ML, Wood LG., Singh H, Moughan, PJ (2006) Means of delivering recommended levels of long chain n-3 polyunsaturated fatty acids in human diets. J Food Sci 71: 66-71

Geelen A, Brouwer IA, Zock PL, Katan MB (2004) Antiarrhythmic effects of n-3 fatty acids: evidence from human studies. Curr Opin Lipidol 15:25-30

Hermoso-Diaz IA, Velázquez-González MA, Lucio-Garcia MA, Gonzalez-Rodriguez JG (2014) A Study of *Salvia hispanica* as Green Corrosion Inhibitor for Carbon Steel in Sulfuric Acid. Chem Sci Rev Lett 3: 685-697

Hipsley EH (1953) Dietary “fibre” and pregnancy toxaemia. Brit med J 2(4833) 420.

Huang YS, Zhang JT (1991) Antioxidative effect of three water-soluble components isolated from *Salvia miltiorrhiza* in vitro. Yao xue xue bao= Acta Pharm Sin 27: 96-100

Ixtaina VY, Martinez ML, Spotorno V, Mateo CM, Maestri DM, Diehl BWK, Nolasco SM, Tòmas MC (2011) Characterization of Chia seed oils obtained by pressing and solvent extraction. J Food Comp Anal 24:166–174

Jamboonsri W, Phillips TD, Geneve RL, Cahill JP, Hildebrand DF, (2012). Extending the range of an ancient crop, *Salvia hispanica* L. – a new ω3 source. Gen Res Crop Evol 59:171–178

Lairon D, Arnault N, Bertrais S, Planells R, Clero E, Hercberg S, Boutron-Ruault MC (2005) Dietary fiber intake and risk factors for cardiovascular disease in French adults. Am J Clin Nutr 82:1185–1194

Li BQ., Fu T, Dongyan Y, Mikovits J A, Ruscetti FW, Wang JM (2000) “Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry,” Biochemical and Biophysical Research Communications, vol. 276, no. 2, pp. 534–538

Lin KY, Daniel JR, Whistler RL (1994) Structure of Chia seed polysaccharide exudate. Carbohyd Polym 23: 13–18

Liu S, Stampfer MJ, Hu FB, Giovannucci E, Rimm E, Manson JE, Hennekens CH, Willett WC (1999) Whole-grain consumption and risk of coronary heart disease: results from the Nurses’ Health study. Am J Clin Nutr 70:412–419

Lowe ED., Gao GY, Johnson LN, Keung WM (2008) Structure of Daidzin, a Naturally Occurring Anti-Alcohol-Addiction Agent, in Complex with Human Mitochondrial Aldehyde Dehydrogenase. J Med Chem 51: 4482-4487

Lu Y, Foo LY (1999) Rosmarinic acid derivatives from Salvia officinalis. Phytochemistry 51: 91-94

Lu Y, Foo LY (2001) Salvianolic acid L, a potent phenolic antioxidant from *Salvia officinalis*. Tetrahedron Lett 42:8223-8225

Lu Y, Foo LY (2002) Polyphenolics of Salvia—a review. Phytochemistry 59:117-140

Martínez-Cruz O, Paredes-López O (2014) Phytochemical profile and nutraceutical potential of Chia seeds (*Salvia hispanica* L.) by ultra-high performance liquid chromatography. J Chromatogr A 1346:43–48

Matthaus B (2002) Antioxidant activity of extracts obtained from residues of different oilseeds. J Agric Food Chem 50: 3444–3452

Menga V, Amato M, Phillips TD, Angelino D, Morreale F, Fares C (2017) Gluten-free pasta incorporating Chia (*Salvia hispanica* L.) As thickening agent: an approach to naturally improve the nutritional profile and the in vitro carbohydrate digestibility Food Chem Doi: 10.1016/j.foodchem.2016.11.151

Miller HE (1971) A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc 48: 91-105

Mongeau R, Brassard M (1982) Insoluble dietary fiber from breakfast cereals and brans bile salt and water holding capacity in relation to particle size. Cereal Chem 59: 413–417

Montonen J, Knekt P, Jarvinen R, Aromaa A, Reunanen A. (2003) Whole-grain and fiber intake and the incidence of type 2 diabetes. Am J Clin Nutr 77:622–629

Muñoz LA, Cobos A, Diaz O, Aguilera JM (2012a) Chia seeds: Microstructure, mucilage extraction and hydration. J Food Eng 108:216-224

Muñoz LA, Aguilera JM, Rodriguez-Turienzo L, Cobos A, Diaz O (2012b) Characterization and microstructure of films made from mucilage of *Salvia hispanica* and whey protein concentrate. J Food Eng 111: 511-518

Palma F, Donde M, Lloyd WR (1947) Fixed oils of Mexico. Part 1. Oil of Chia—*Salvia hispanica*. J Am Oil Chem Soc 24:27

Parnham MJ, Kesselring K (1985) Rosmarinic acid. Drugs of the Future 10: 756–757

Pearce KN, Kinsella JE (1978) Emulsifying properties of proteins: evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry, 26(3), 716-723.

Peiretti PG, Meineri G (2008) Effects on growth performance, carcass characteristics, and the fat and meat fatty acid profile of rabbits fed diets with Chia (*Salvia hispanica* L.) seed supplements. Meat Science: 80, 4, 1116–1121

Petruzziello L, Iacopini F, Bulajic M, Shah S, Costamagna G (2006) Review article: uncomplicated diverticular disease of the colon. Aliment Pharmacol Ther 23:1379–1391

Porras-Loaiza P, Jiménez-Munguía MT, Sosa-Morales ME, Palou E, López-Malo A (2014) Physical properties, chemical characterization and fatty acid composition of Mexican chia (*Salvia hispanica* L.) seeds. Int J Food Sci Tech 49: 571–577

Reyes-Caudillo E, Tecante A, Valdivia-López MA (2008) Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican Chia (*Salvia hispanica* L.) seeds. Food Chem 107: 656–663

Sargi SC, Silva BC, Santos HMC, Montanher PF, Boeing JS, Júnior S, Visentainer JV (2013) Antioxidant capacity and chemical composition in seeds rich in omega-3: Chia, flax, and perilla. Food Sci Technol (Campinas) 33:541-548

Segura-Campos MR, Ciau-Solìs N, Rosado-Rubio G, Chel-Guerrero L, Betancour-Ancona D (2014) Physicochemical characterization of Chia (*Salvia hispanica*) seed oil from Yucatan, México. Agric Sci 5:220–226

Steffen LM, Jacobs DR Jr, Stevens J, Shahar E, Carithers T, Folsom AR (2003) Associations of whole-grain, refined grain, and fruit and vegetable consumption with risks of all-cause mortality and incident coronary artery disease and ischemic stroke: the Atherosclerosis Risk in Communities (ARIC) Study. Am J Clin Nutr 78:383–390

Taga MS, Miller EE, Pratt DE (1984) Chia seeds as a source of natural lipid antioxidants. J Am Oil Chem Society 61: 928–932

Tolba R, Guosheng W, Aicheng C (2011) "Adsorption of dietary oils onto lignin for promising pharmaceutical and nutritional applications." BioResources 6.2: 1322-1335

Trowell HC, Southgate DAT, Wolever TMS, Leeds AR, Gassull MA, Jenkins DJ (1976) A. Dietary fiber redefined. Lancet 307(7966), 967

Vázquez-Ovando A, Rosado-Rubio G, Chel-Guerrero L, Betancur-Ancona D (2009) Physicochemical properties of a fibrous fraction from Chia (*Salvia hispanica* L.). LWT-Food Sci Technol 42: 168–217

Vuksan V. Whitham D, Sievenpiper JL, Jenkins AL, Rogovik AL, Bazinet RP, Vidgen E, Hanna A (2007) Supplementation of conventional therapy with the novel grain Salba (*Salvia hispanica* L.) improves major and emerging cardiovascular risk factors in type 2 diabetes. Diabetes Care 30: 2804-2810

Vuksan V, Jenkins AL, Dias AG, Lee AS, Jovanovski E, Rogovik AL, Hanna A (2010) Reduction in postprandial glucose excursion and prolongation of satiety: possible explanation of the long-term effects of whole grain Salba (*Salvia Hispanica* L.)." Eur J Clin Nutr 64 : 436-438

Wall R, Ross RP, Fitzgerald GF, Stanton C. (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 68:280-9

Weber CW, Gentry HS, Kohlhepp EA, McCrohan PR (1991) The nutritional and chemical evaluation of Chia seeds. Ecol Food Nutr, 26: 119-125

Whelton SP, Hyre AD, Pedersen B, Yi Y, Whelton PK, He J. (2005) Effect of dietary fiber intake on blood pressure: a meta-analysis of randomized, controlled clinical trials. J Hypertens 23:475–481

Wu Y.J, Hong CY, Lin SJ, Wu P, Shiao MS. (1998) Increase of vitamin E content in LDL and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of *Salvia miltiorrhiza*. Arterioscle Thromb Vasc Biol 18: 481-486

Yagi A, Fujimoto K, Tanonaka K, Hirai K, Takeo S (1989) Possible active components of Tan-Shen for protection of myocardium against ischemia. Planta med 55:51-54

Yao L H, Jiang YM, SHI J, Tomas-Barberan FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. Plant Food Hum Nutr 59: 113-122

Yeh H, Su N, Lee M (2005) Chemical compositions and physicochemical properties of the fiber-rich materials prepared from shoyu mash residue. J Agr Food Chem 53: 4361–4366

Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S (2011) "Antiviral activity of four types of bioflavonoid against dengue virus type-2" Virology Journal, vol. 8, article 560,

Zhang HJ, Li LN (1994) Salvianolic acid I: a new depside from *Salvia cavaleriei*. Planta Med 60: 70–72

Zhao BL, Jiang W, Zhao Y, Wu JH, Xin WJ (1996) Scavenging effects of *Salvia miltiorrhiza* on free radicals and its protection for myocardial mitochondrial membranes from ischemia-reperfusion injury. Biochem Mol Biol Int 38: 1171–1182

Zhu W, Jia Q, Wang Y, Zhang Y, Xia M (2012) The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP–PKA-dependent signaling pathway. Free Radical Bio Med 52:314-327

Zou ZW, Xu LN, Tian JY (1992) Antithrombotic and antiplatelet effects of rosmarinic acid, a water-soluble component isolated from radix *Salviae miltiorrhizae* (danshen). Yao xue xue bao = Acta pharmaceutica Sinica, 28: 241-245

5. Metabolomic Analysis of *Salvia hispanica* Seeds Using NMR Spectroscopy and Multivariate Data Analysis§

5.1 Introduction

The re-discovery of chia (*Salvia hispanica* L.), an ancient crop from Central America, dates back to the 1990s, when Gentry et al. (1990) and Coates and Ayerza (1996) pointed out the high content of ω-3 in its fruits (commonly called seeds). The plant belongs to the *Lamiaceae* family, and originates between Mexico and Guatemala (Cahill 2004). Very recently, Bochicchio et al. (2015a) reviewed findings on genetics, quality, uses and agronomy of the crop. In particular, most of the published papers addressed the amount and composition of fatty acids in chia oil in their area of origin (Ayerza 1995; Ayerza and Coates 2011; Segura-Campos et al., 2014) or in new areas such as Africa (Yeboah et al., 2014) and Europe (Amato et al., 2015), and showed that the plant is one of the richest ω -3 seed source in nature.

Seeds are also an important source of protein, with reports of 12 to 26% in whole seeds (Ayerza and Coates 2004, 2009a, b, 2011, Capitani et al., 2012). A high amount of dietary fiber is also reported (e.g. chia meal contains 33.9–39.9% of dietary fiber, Capitani et al., 2012). Seeds are rich in vitamin B (Bushway et al., 1984) and in antioxidant compounds such as tocopherols, phytosterols, carotenoids, and phenolics. These include chlorogenic and caffeic acids, myricetin, quercetin, and kaempferol (e.g., Reyes-Caudillo et al., 2008; Marineli et al., 2014; Amato et al., 2015). Because of their unique chemical composition, the seeds are also used in

§ de Falco, B., Incerti, G., Bochicchio, R., Phillips, T.D., Amato, M., & Lanzotti, V. (2017). Metabolomic Analysis of *Salvia hispanica* Seeds Using NMR Spectroscopy and Multivariate Data Analysis. *Industrial Crops and Products,* 99: 86-96.

cosmetics (Muñoz et al. 2013) and in ethnic medicine for the treatment of eye infections (Lu and Foo 2002; Reyes-Caudillo et al., 2008).

Moreover, Hermoso-Diaz et al. (2014) found that a methanol extract of *S. hispanica* can be a good green corrosion inhibitor for carbon steel in sulfuric acid, and therefore the plant is a promising substitute for toxic organic inhibitors. Authors attributed corrosion inhibition to the formation of a protective barrier film by active components of the extracts adsorbed on the surface of carbon steel inside oxide layer via the lone pairs of electrons present on their oxygen-bearing functional groups, OH and COOH. To this purpose, linoleic and α-linolenic acids were identified as the most likely compounds involved in the inhibition mechanism (Hermoso-Diaz et al., 2014).

The research on leaves composition highlighted the presence of essential oils (Ahmed et al., 1994), polyunsaturated fatty acids (PUFA), proteins and fiber (Peiretti and Gai, 2009). Amato et al. (2015) found flavonoids in the leaves and reported in addition the presence of two uncommon compounds, acetyl vitexin and acetyl orientin.

The center of origin and of genetic and phenotypic diversity of chia populations ranges from semi-temperate and temperate highlands of western Mexico to the trans-volcanic belt and Puebla, between 1,400 and 2,200 metres above sea level. (m.a.s.l.) (Cahill 2004; Hernandez-Gomez and Miranda-Colın 2008; Miranda-Colin 1978). It is originally a short-day flowering specie; therefore, it can viably produce seeds in a restricted range of latitudes. Nevertheless, breeding efforts have produced longer-day flowering genotypes in order to extend the range of this crop to other temperate areas (Jamboonsri et al., 2012). Most of these new lines are mutants capable of flower induction between 13 and 16 h of daylength and a few are day-length insensitive. As the growing area and the market of the crop expand, there is interest in further identification of seed metabolites, and in characterizing possible differences due to genotype and crop management.

Thus, we have undertaken a comparative analysis of the seeds of commercial short-day flowering black and white genotypes and the long-day and early flowering mutants G3, G8, G17 and W13.1. The study was also extended to the seeds of two black populations grown at different nitrogen supply.

The analysis was done by using a metabolomic approach based on the identification and quantification of all metabolites in the plant material by ^{1}H NMR spectroscopy, followed by multivariate data analysis (Verpoorte et al., 2007, Kim et al., 2010, Incerti et al., 2013, de Falco et al., 2015).

This work aims at evaluating the chemical composition of commercial and mutant genotypes of chia seeds through the identification of the major classes of organic compounds by NMR analysis and from a quantitative point of view through integration of the NMR spectra followed by chemometrics. The effect of mineral fertilization, on the metabolome of field-grown chia was also evaluated. Results demonstrate the usefulness of ^{1}H NMR coupled with chemometrics for sample classification according to seed source.

5.2 Materials and methods

5.2.1 Chemicals

First-grade dichloromethane and methanol were purchased from Delchimica Scientific Laboratories Glassware (Naples, Italy). Deuterium oxide (99.8 atom %D) and dimethyl-4-silapentane sodium sulfonate (DSS) was obtained from ARMAR Chemicals (Switzerland), chloroform-d (99.8 atom %D) contains 0.03% (v/v) TMS was purchased from Sigma-Aldrich (Italy). Pure standard amino acids, caffeic acid, chlorogenic acid, genistein and quercetin were used as references (Sigma-Aldrich, Italy).

5.2.2 Seeds obtained with different nitrogen supply

Samples of *Salvia hispanica* L. were grown in 2013 at Masserie Saraceno (Atella - PZ, Southern Italy, Lat. N 40° 51' 37.59", Long. E 15° 38' 49.43") on a Luvi-vertic Phaeozem (Iuss working group, 2006) loamy clay soil periodically amended with the solid fraction of on-farm biogas-digested materials. Before sowing the soil, chemical characteristics were: pH 6.8; N 1.9 g kg^{-1}; phosphates (P_2O_5) 50.3 g kg^{-1}; potassium oxide (K_2O) 1430 g kg^{-1}. Rainfall during the growing period was 322.4 mm and temperature minimum, maximum and mean during the period were 38.1, −2.3 and 16.9 °C, respectively. Black *S. hispanica* commercial seeds (B_{2012}) available at Eichenhain (Hofgeismar - DE) were sowed on 21/06/2013 and grown with non-limiting water supply (drip irrigation). A field randomized block design with three replications was established to test two strategies of mineral nitrogen fertilization: B_0 (no mineral fertilization); B_M (mineral supplement fertilization 60 days after sowing with NH_4NO_3 at 20 kg ha^{-1} of N).

5.2.3 Commercial and long-day flowering chia

Two black (B_{2012} and B_{2013}) and one white (W) chia commercial seeds were purchased from Eichenhain (Hofgeismar - DE). Four long-day mutant genotypes, of which three black (G3, G8, G17) and one white (W13.1) obtained as described in Jamboonsri (2010) and Jamboonsri et al. (2012) were made available to the University of Basilicata through an agreement with the University of Kentucky. All seeds were grown in triplicate in 0.35 m diameter pots with 10:30:60 w:w:w vermiculite, sand and wood litter compost. Plants were grown with non-limiting water supply and fertilized with commercial potting liquid fertilizer with a total amount corresponding to 0.53 g $plant^{-1}$ N.

5.2.4 Extraction procedure

Seeds were dried at room temperature and powdered finely with a pestle in a mortar. The extraction of all metabolites was made according to the procedure previously applied (Incerti et al., 2013). The sample (300 mg, each) was dissolved in 10 ml of $CH_2Cl_2/MeOH/H_2O$ (2:1:1), mixed by vortex and incubated 15 minutes at room temperature. To ensure efficient lysis of cell membranes and to promote the escape of all metabolites, solution was sonicated for 1 minute at 25 °C with a Bandelin Sonoplus HD 2070. After centrifugation (5000 rpm, 30 minutes, 25 °C), the aqueous and organic fractions were accurately separated, with particular attention to discard the interphase. The extraction and subsequent centrifugation were repeated a second time and solvents evaporated to dryness under vacuum (Rotavapor R-114, Büchi, Switzerland) keeping the temperature at 30 °C to inhibit the decomposition of thermolabile compounds. The dried samples were stored at 4 °C until analysis. All experiments were performed in triplicate to assure their reproducibility.

5.2.5 Spectroscopic analysis

^1H NMR spectra were acquired both on the aqueous and organic fractions: 600 µl of deuterium oxide (99.8% D_2O) was used to solubilize the aqueous fractions while chloroform-d (99.8% $CDCl_3$) was added to the organic fractions and transferred into a 5 mm NMR tube following the protocol developed by Choi et al. (2004, 2005). DSS and TMS were used as an internal standard for aqueous and organic fractions, respectively. In particular, the pH of aqueous fractions was adjusted to 6.0 by using KH_2PO_4 as a buffering agent and 1N NaOD (Choi et al., 2004, 2005). All spectra were acquired at 298 K with Varian Unity Inova spectrometer operating at a ^1H frequency of 400.422 MHz. The recycle time was set to 5 s, and a 45 pulse angle was used. Chemical shifts were referred to DSS and TMS signals (both δ 0.00 ppm). All spectra were processed using iNMR program (www.inmr.net), phased and baseline corrected

manually. Quantification was performed by signal integration relative to the internal standard, DSS and TMS. The region of the solvent peaks was excluded from the analysis. Spectral peak assignments of fatty acids, organic acids, amino acids, carbohydrates, choline, the flavonoids genistein and quercetin, the caffeoyl derivatives caffeic acid, chlorogenic acid and rosmarinic acid were obtained on the basis of pure standards purchased by Sigma-Aldrich. Spectral peak assignments of these and the other detected metabolites were obtained by two-dimensional (2D) NMR experiments, including ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{13}C heteronuclear single-quantum correlation (HSQC) and comparison with data reported in the literature (Dai et al., 2010; Jiang et al., 2014; Mattoli et al., 2006; Brahmi et al., 2015). Tanshinone I and 15,16-dihydrotanshinone were detected in the aqueous fractions and the spectral peak assignments obtained by comparison of the NMR data with those of pure standards purchased by Sigma-Aldrich. The COSY spectra were acquired with a spectral width of 6130 Hz in both dimensions, 8K data points, and 512 increments with 32 transients per increment. The HSQC spectra were acquired with spectral widths of 8000 Hz in the F2 dimension and 25000 Hz in the F1 dimension, a data matrix with a size of 1K × 256 data points, and 64 transients per increment. All spectra were manually phased and baseline corrected.

5.2.6 Multivariate data analysis

Multivariate analysis were applied to ^{1}H NMR spectral data from both polar and organic extracts of seeds, leaving out signals from residual solvent. ^{1}H NMR spectra were preliminarily normalized and reduced to integrated regions of equal widths (bins = 0.01 ppm), corresponding to 0 – 10 ppm and subsequently reduced to ASCII files using the software package iNMR v. 5.1.2 (Mestrelab Research, Molfetta, Italy). Peak quantification was performed by signal integration relative to the internal standard, with peak intensity expressed as parts per thousand with respect to the whole spectrum once the region of the residual solvent peaks was excluded.

For general, exploratory purpose, two data matrices were considered, for aqueous and organic extracts respectively, including peak data from the whole ^{1}H NMR spectra, averaged over the replicated spectra for each studied population. Each data matrix was submitted to Principal Component Analysis (PCA) ordination using the STATISTICA 7 Software (StatSoft Inc., Tulsa, Oklahoma, USA). In a more detailed PCA analysis on spectral data from the polar fraction, a submatrix was considered, obtained by excluding spectral data from the resonance region δ 3.2-4.1, corresponding to carbohydrates, the most abundant compounds in the samples. In this way, limited to less abundant polar compounds, it was possible to explore overall chemical differences among populations.

5.2.7 Chemometrics

Quantitative data from both polar and organic extracts of chia seed samples were compared through one-way analysis of variance, followed by post-hoc Tukey's HSD test at $p<0.05$. The analysis was separately performed for each metabolite identified in the replicated samples, using spectral signals reported in Table 5.1, under the null hypothesis that the amount of single metabolites was not different among lines. Effects of supplemental mineral nitrogen fertilization were separately tested for single metabolites, comparing mean amounts in B_M and B_0 seeds by two-tails Student's t test for independent samples. In order to control for type I statistical error due to multiple comparisons, the threshold value of p for statistical significance was set to $\alpha' = 0.05/N = 0.00156$, with N=32 being the number of comparisons (i.e. metabolites simultaneously tested), following the Bonferroni's correction method; p-values ranging between 0.00156 and 0.05, since possibly affected by Type I statistical error, were considered marginally significant. All statistics were calculated using the Software STATISTICA 7 (StatSoft Inc., Tulsa, Oklahoma, USA).

5.3 Results and discussions

5.3.1 Metabolite profile

In the present study, the metabolome of different genotypes from chia (*Salvia hispanica* L.) seeds was analyzed. While the ^{1}H NMR spectra of organic extracts mostly showed unsaturated fatty acids as the major compounds (Fig. 5.1A, Table 5.1), the spectra of the aqueous extracts showed the presence of metabolites belonging to different chemical classes (Fig. 5.1B, Table 5.1). Their identification is showed on the ^{1}H NMR spectrum of B_{2012}, taken as model (Fig. 5.2) and described hereafter, with reference to specific diagnostic chemical shift values from Table 5.1.

5.3.2 Organic acids

Several organic compounds were identified in the ^{1}H NMR spectrum of the aqueous extracts (Table 5.1). In particular, the presence of lactic acid was confirmed by its methyl group resonating at δ 1.20 as a doublet with a J of 7 Hz. The characteristic signals of citric acid due to the methylene hydrogens (AB spin system) were clearly visible at δ 2.63 and 2.79 with a coupling constant of 17.5 Hz. Singlets at δ 2.41 and 3.16, relative to CH_2 between two carboxyl groups, were identified in the spectrum and attributed to succinic and malonic acid, respectively. A further singlet, resonating in the low field region of the spectra at δ 8.48, indicated the presence of formic acid. The identification and spectral assignments of these metabolites was obtained by comparison with the NMR data of standard organic acids (see Materials and methods). To the best of our knowledge, this is the first report on the detection of these compounds in *S. hispanica* seeds.

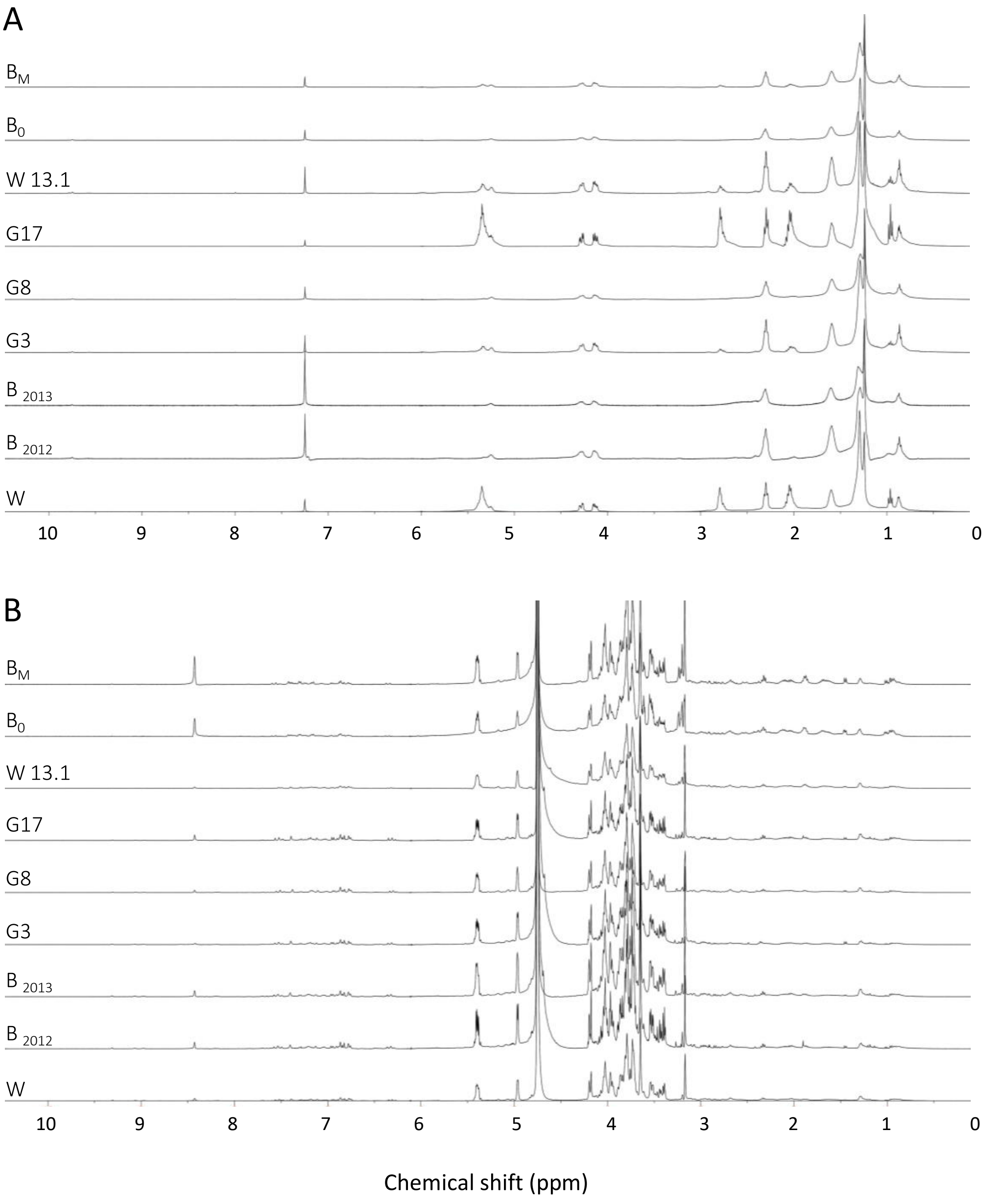

Fig. 5.1 ^{1}H NMR spectra at 400 MHz of the seeds of nine chia (*Salvia hispanica* L.) populations. A) spectra in $CDCl_3$ at 400 MHz. Spectral regions where no peaks were observed are not indicated, and reported as scale breaks on the x-axis. B) spectra in D_2O solvent; the vertical scale in the spectral regions from 0.5 to 3.0 ppm and from 5.5 to 10 ppm is expanded five times for better visibility.

5.3.3 Free amino acids

In the ^{1}H NMR spectra of the aqueous extracts of the analyzed seeds several free amino acids were identified by their diagnostic chemical shift values (Table 5.1). In the region ranging from δ 0.91 to 1.10, signals related to the methyl groups of the aliphatic amino acids leucine, isoleucine and valine were observed. In addition, the signal of the alanine β-CH_3 resonated at δ 1.45 as a doublet (J = 7 Hz) for its proximity to the amino group. Further diagnostic signals included those found for γ-aminobutyric acid resonating at δ 2.33 and 2.98 (both t, J = 7 Hz), and for the α-CH of aspartic acid resonating at 2.84 ppm (dd, J = 4.0; 18.0 Hz). This methodology does not allow to identify all free amino acids present in the extract because the signals of many of them were overlapped in the spectra. The identification and spectral assignments of the detected amino acids was confirmed by comparison with the NMR data of standard amino acids (see Materials and methods). However, previous report by Ayerza (2013) on the analysis of seeds, genotypes Tzotzol and Iztac, by classical methods identified all the free amino acids, including those here reported.

5.3.4 Carbohydrates

In the spectra of seeds aqueous extracts, overlapped signals for sugar protons were observed from 3.40 to 4.10 ppm, that is a very crowded region of the ^{1}H NMR spectra. Outside such region, diagnostic anomeric proton signal (H1) of glucose was identified at δ 4.96 (d, J = 4.0 Hz), while the anomeric protons of sucrose and raffinose appeared at δ 5.38 and 5.40 respectively (both d, J = 4.0 Hz). The identification and spectral assignments of glucose, sucrose and raffinose (Table 5.1) have been obtained by comparison with NMR data of standard sugars (see Materials and methods). This is the first report on the analysis of carbohydrates from *S. hispanica* seeds.

Table 5.1 ^{1}H-NMR chemical shifts, assignment and multiplicity at 400 MHz of organic compounds found in extracts of chia seeds in D_2O (A) and in $CDCl_3$ (B).

Compounds	Assignment	^{1}H (ppm)	Multiplicity [*J* (Hz)]	Compounds	Assignment	^{1}H (ppm)	Multiplicity [*J* (Hz)]
A) D_2O extracts							
Organic acids				***Caffeoyl derivatives***			
Acetic acid (AC)	CH_3	1.91	s	Caffeic Acid (CA)	CH-8	6.34	d [16.0]
Citric acid (CI)	α,γ-CH	2.63	d [17.5]		CH-5	6.76	d [8.0]
	α^{I},γ^{I} -CH	2.79	d [17.5]		CH-6	6.94	dd [2.0, 8.0]
Malonic acid (MA)	CH_2	3.16	s		CH-2	7.20	d [2.0]
Lactic acid (LA)	CH_3	1.20	d [7.0]		CH-7	7.50	d [16.0]
Succinic acid (SU)	α,β-CH_2	2.41	s	Chlorogenic acid (Chlo)	CH-8^{I}	6.32	d [16.0]
Formic acid (FO)	HCOOH	8.48	s	5-caffeoylquinic acid	CH-5^{I}	6.95	d [8.0]
Fumaric acid (FU)	α,β -CH=CH	6.49	s		CH-6^{I}	7.04	d [2.0, 8.0]
Amino acids					CH-2^{I}	7.16	d [2.0]
Alanine (Ala)	β-CH_3	1.45	d [7.0]		CH-7^{I}	7.55	d [16.0]
γ-aminobutyric acid (GABA)	β-CH_2	1.90	m	Rosmarinic Acid (RO)	CH-7^{I}a	2.97	dd [10.0, 14.0]
	α-CH_2	2.33	t [7.0]		CH-7^{I}b	3.06	dd [4.0, 14.0]
	γ-CH_2	2.98	t [7.0]		CH-8	6.30	d [16.0]
Aspartic acid (Asp)	β-CH_2	2.71			CH-6^{I}	6.76	dd [2.0, 8.0]
	α-CH	2.84	dd [4.0, 18.0]		CH-5^{I}	6.81	d [7.8]
Isoleucine (Ile)	δ-CH_3	0.91	t [7.0]		CH-5	6.82	d [8.0]
	γ^{I}-CH_3	1.01	d [7.0]		CH-2^{I}	6.88	d [3.0]
Leucine (Leu)	δ-CH_3	0.95	d [7.0]		CH-6	7.00	dd [2.0, 8.0]
N-acetylglutamic acid (NAcGlu)	CH_2	1.90			CH-2	7.11	d [2.0]
	-$COCH_3$	2.05			CH-7	7.51	d [16.0]
	γ-CH_2	2.33	t [7.0]	***Other compounds***			
Proline (Pro)	γ-CH_2	2.08	m	choline (Cho)	$N(CH_3)^{3+}$	3.19	s
	CH_2	2.29	m	15,16-Dihydrotanshinone	CH-17	1.37	d [7.0]
Valine (Val)	γ^{I}-CH_3	0.99	d [7.0]		CH-18	2.62	s
	γ-CH_3	1.01	d [7.0]		CH-3	7.37	d [7.0]
Carbohydrates					CH-2	7.50	dd [8.0, 7.0]
Glucose (Glc)	CH-4	3.25			CH-7	7.83	d [8.0]
	CH-2	3.53			CH-6	8.22	d [8.0]
	CH-5	3.83			CH-1	9.19	d [8.0]
	CH-3	4.65			CH.17	2.28	
	CH-1	4.96	d [4.0]	Tanshinone I	CH-18	2.62	s
Raffinose (Raff)	Glc-C_2H	3.55			CH-3	7.32	d [7.0]
	Glc-C_3H	3.78			CH-2	7.50	dd [8.0, 7.0]
	Glc-C_5H	4.08			CH-7	7.82	d [8.0]
	Glc-C_1H	5.40	d [4.0]		CH-6	8.18	d [8.0]
Sucrose (Sucr)	Glc-C_2H	3.55			CH-1	8.98	d [8.0]
	Glc-C_3H	3.82					
	Glc-C_1H	5.38	d [4.0]	**B) $CDCl_3$ extracts**			
Flavonoids				Fatty acids	ω_1-CH_3	0.89	
Genistein (GE)	CH-6	6.30	d [2.0]		ω_3-CH_3	0.97	t [8.0]
	CH-8	6.46	d [2.0]		-$(CH_2)_n$-	1.28	
	CH-3^{I}, 5^{I}	7.04	d [8.0]		γ-CH_2	1.61	
	CH-2^{I}, 6^{I}	7.44	d [9.0]		allylic C	2.07	m
	CH-2	8.29	s		β-CH_2	2.32	t [7.0]
Quercetin (QUE)	CH-6	6.03	d [2.0]		diallylic C	2.81	
	CH-8	6.50	d [2.0]	glycerol	CH-1, 3	4.22	
	CH-5^{I}	7.02	d [8.0]	MUFA	CH=CH	5.28	
	CH-6^{I}	7.69	dd [2.0, 8.0]	PUFA	CH=CH	5.37	

5.3.5 Flavonoids

As reported in the literature (Taga et al., 1984), the content of flavonoids, such as genistein and quercetin (Fig. 5.3), is very low in chia seeds. However, signals for genistein at δ 6.46 (CH-8, d, 2 Hz) and 8.29 (CH-2, s) were still visible in the spectra (Fig. 5.2), while characteristic signals of quercetin were present at δ 6.50 (CH-8, d, 2 Hz) and δ 7.69 ($CH-6^I$, dd, 2.0, 8.0 Hz) (Table 5.1). The identification of genistein and quercetin was confirmed by comparison with NMR data of standard flavonoids (see Materials and methods).

5.3.6 Polyphenols

It is well known that *S. hispanica* seeds contain a large number of polyphenolic compounds which are constructed from the caffeic acid building block (Marineli et al., 2014; Martínez-Cruz et al., 2014). In the ^1H NMR spectra of aqueous extracts, diagnostic signals of caffeic acid derivatives were detected in the low-field region. In particular, signals at δ 6.30, 6.32, 6.34 (CH-8, $CH-8^I$) and δ 7.51, 7.55, 7.50 (CH-7, $CH-7^I$) resonating all as doublets with a coupling constant of 16 Hz, indicated the presence of the *trans* hydroxyl-cinnamoyl vinyl groups belonging to rosmarinic, chlorogenic and caffeic acids (Fig. 5.3). Moreover, the presence of double doublets at δ 6.76, 6.95 and 7.04 confirmed the typical spin system of the aromatic ring of caffeoyl moiety. The identification of caffeic, chlorogenic and rosmarinic acids was confirmed by comparison with NMR data of standards (see Materials and methods). The presence of 5-caffeoyl quinic acid have been proposed on the basis of 2D COSY and HSQC data.

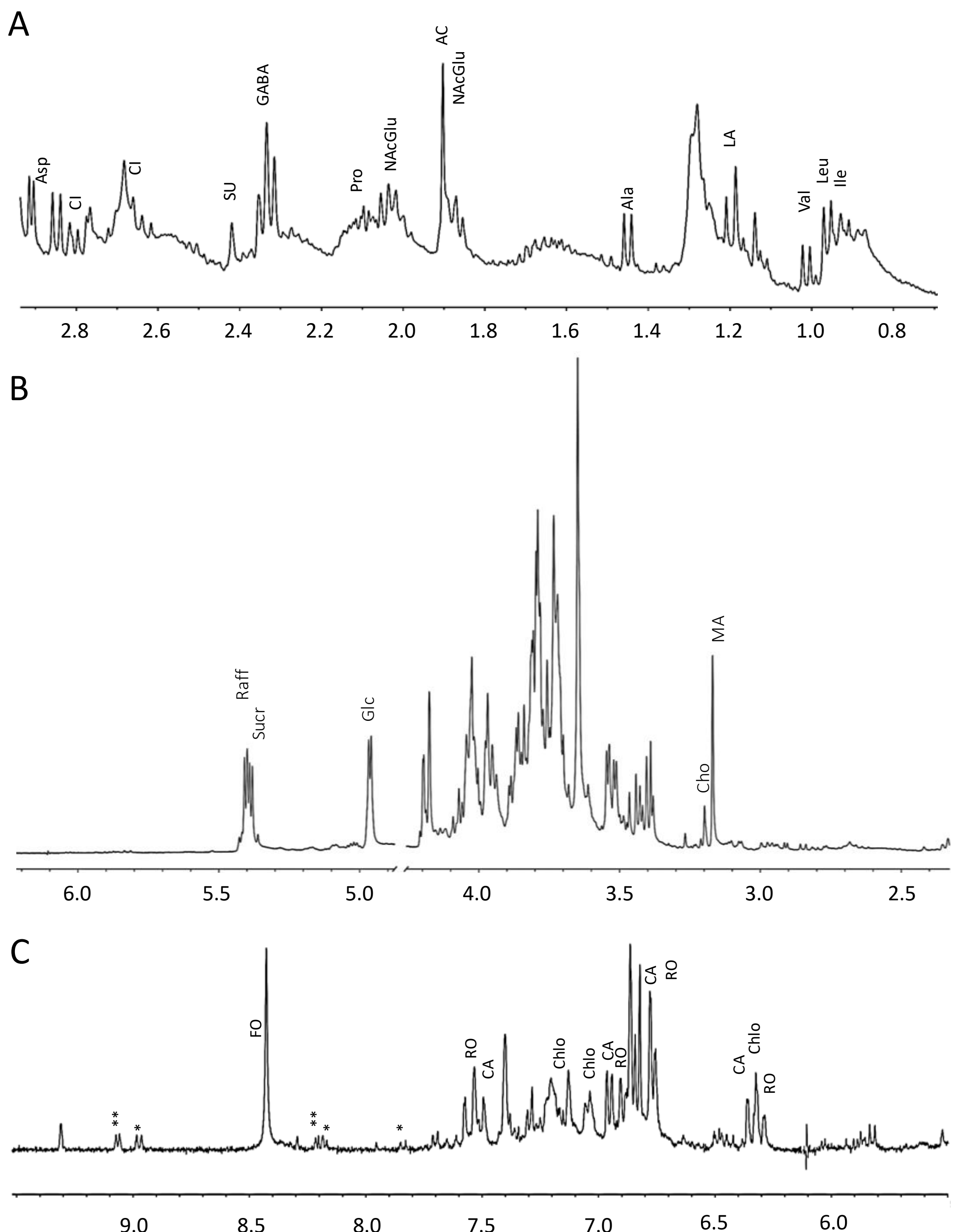

Fig. 5.2 ^{1}H NMR spectrum (D_2O, 400 MHz) of B_{2012} with identification of the compounds detected. A) spectral region from 0.7 to 2.9 ppm; B) spectral region from 2.9 to5.7; C) spectral region from 5.7 to 10 ppm. Vertical scale in A and C is expanded five times for better visibility. * Possible signals for tanshinone I. ** Possible signals for15,16-dihydrotanshinone.

5.3.7 Other compounds

In addition to the polar compounds described above, ^{1}H NMR spectra from the polar extracts of seeds suggested the presence of choline identified by comparison with pure standards (see Materials and methods). In addition, Tanshinone I {1,6-dimethylnaphtho[1,2-g][1]benzofuran-10,11-dione} and 15,16 dihydro Tanshinone I (Fig. 5.3) have been detected because of their characteristic signals in the low field region of the spectrum (Table 5.1) in comparison with NMR data of pure standards, as reported in Materials and methods. While such molecules were previously found in roots of *Salvia miltiorrhiza* (Dai et al., 2010), this is the first report showing their occurrence in chia seeds.

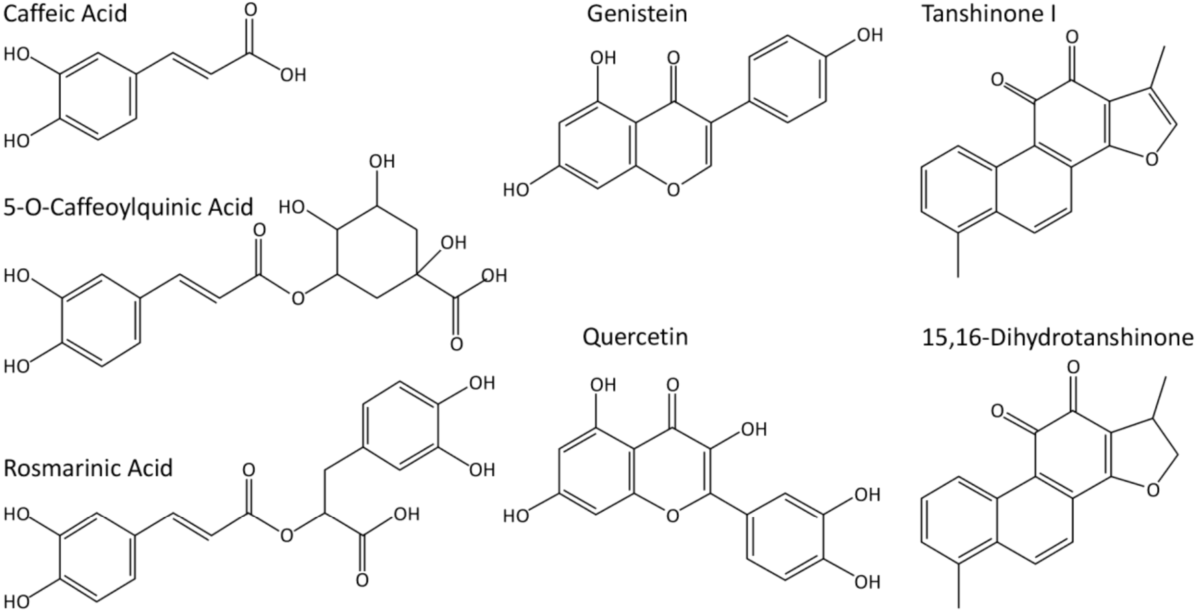

Fig. 5.3 Chemical structures of characteristic compounds found in chia seeds.

5.3.8 Fatty acids

^{1}H NMR spectra were also acquired on the organic extracts of all samples (Fig. 5.1A). According to others published data, Amato et al., (2015) reported that the apolar fraction is mainly composed of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Their diagnostics chemicals shifts were shown in the Table 5.1. In particular, terminal

methyl group protons were detected at δ 0.89, excepted for α-linolenic acid (18:3; ALA) whose methyl group resonates at δ 0.97 due to proximity of the omega-3 double bond. The proton signals attached to the allylic carbons were also identified (δ 2.07). While, the diagnostics peaks of protons attached of diallylic carbons resonating at 2.81 ppm which confirmed the presence of PUFA. Furthermore, olefinic protons were identified at δ 5.28-5.37. Identification and peak assignments of fatty acids (Table 5.1) have been obtained by comparison with NMR data of standards (see Materials and methods).

5.3.9 Exploratory multivariate analysis

The Principal Component Analysis (PCA) based on proton nuclear magnetic resonance from apolar and aqueous fractions produced opposite results, in terms of differences among the studied populations. Concerning the apolar fraction, PCA of all spectral signals from $CDCl_3$ extracts of chia seeds showed a certain similarity among them. All chias were negatively related with the first PC axis, which explained 94.5% of the total variance in the dataset (Fig. 5.4), consistent with high peak of signals corresponding to fatty acids (Table 5.1). Minor differences among the samples were recorded in the relative content of these compounds, with the second PC axis, which separated W and G17 seeds from the others, only explaining a residual 3.7% of the total variance (Fig. 5.4).

On the contrary, the PCA plot of the ^{1}H NMR spectral data from the aqueous fractions (Fig. 5.4) clearly separated the samples along the first ordination axis, which explained 59% of the total variance and was negatively related to all protons resonating in the spectral region from δ 3.2 to 4.1. These signals correspond to carbohydrates, which were overall most abundant in commercial black populations placed at the leftmost of the ordination space (B_{2012} and B_{2013}), while commercial white and long-day flowering mutants placed at the rightmost of the space (W, G8, G17 and W13.1) showed a lower content of these metabolites. G3 showed an

intermediate pattern. The result on black grown in the field in Basilicata showed that the non-fertilized B_0, compared to the fertilized B_M, was closer to commercial black grown in pots (B_{2012} and B_{2013}), thus indicating a lower content of total carbohydrates in seeds due to fertilization.

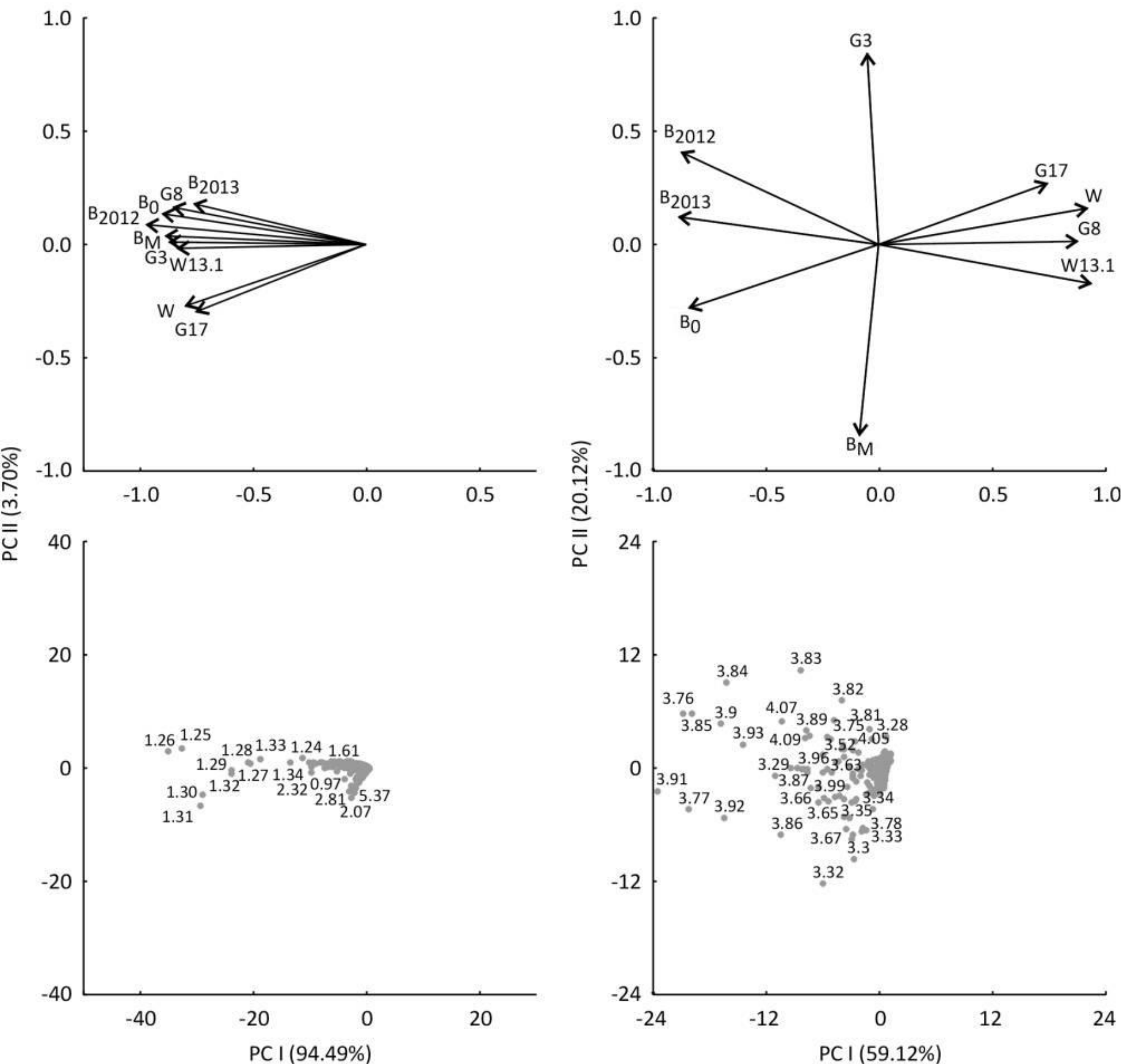

Fig. 5.4 Principal Component Analysis (PCA) of seeds from nine chia populations based on ^{1}H NMR spectra from $CDCl_3$ (left) and D_2O (right) fractions. Top: vector loadings of chia seeds; bottom: factorial scores of resonance intervals of 0.01 ppm. Explained variance of principal components is reported in brackets on the axis labels.

The second ordination axis, explaining 20% of the total variance, was positively and negatively related to the protons resonating at δ 3.83 and 3.32. Even though in these spectral regions different overlapping signals for sugar protons were observed (Fig. 5.1B, 5.2B), that at δ 3.82

is diagnostic of the CH-5 of glucose (Table 5.1). Hence, the distribution of chia population along the second ordination axis, with G3 and B_M clearly separated from the others, and placed at top and bottom of the ordination space, respectively, is consistent with the content of glucose in their seeds, highest in G3 and lowest in fertilized B_M. When excluding main diagnostic signals (i.e. δ 3.2-4.1) from the analysis of ^{1}H NMR spectra from the aqueous fractions, the PCA based on the remaining spectral signals showed restricted regions diagnostic of specific compounds characterizing chia populations (Fig. 5.5). The first ordination axis was negatively related to signals diagnostic of formic acid (δ 8.48, s), and of anomeric proton of raffinose (δ 5.40, d, J = 4.0 Hz) and sucrose (δ 5.38, d, J = 4.0 Hz). The same signals were positively and negatively related to the second ordination axis, respectively. The relative abundance of the corresponding metabolites separated the seeds grown in the field (B_0 and B_M) from those grown in pots. Indeed, B_0 and B_M, placed at the left-bottom of the ordination space, compared to the other populations, showed higher content of formic acid, as well as of other organic acids, and lower content of raffinose and sucrose (Table 5.2). Notably, these two populations, differing only by the fertilization treatment, were relatively close in the ordination space, indicating that their metabolic fingerprinting, except the above-mentioned production of carbohydrates, were relatively similar. Differently, the populations placed at the top of the ordination space (B_{2012}, B_{2013} and G3) showed high content of raffinose and sucrose, being also separated by the content of formic acid and organic acids (low in G3, placed at the top-left of the ordination space, intermediate in B_{2012}, B_{2013}, placed at the top-right). G8, G17, W and W13.1 were all positively related to the first ordination axis. However, their metabolic fingerprinting was different, with G8 and W showing lower content of organic acids and higher content of sucrose and raffinose, and vice-versa for G17 and W13.1. Spectral signals diagnostic of other compounds, such as caffeoyl derivatives and some amino acids (compare Fig. 5.5 with Table 5.1), were less related to the first two principal components compared to carbohydrates and organic acids. However,

the relative abundance of many different compounds was different among chia populations, as hereafter described.

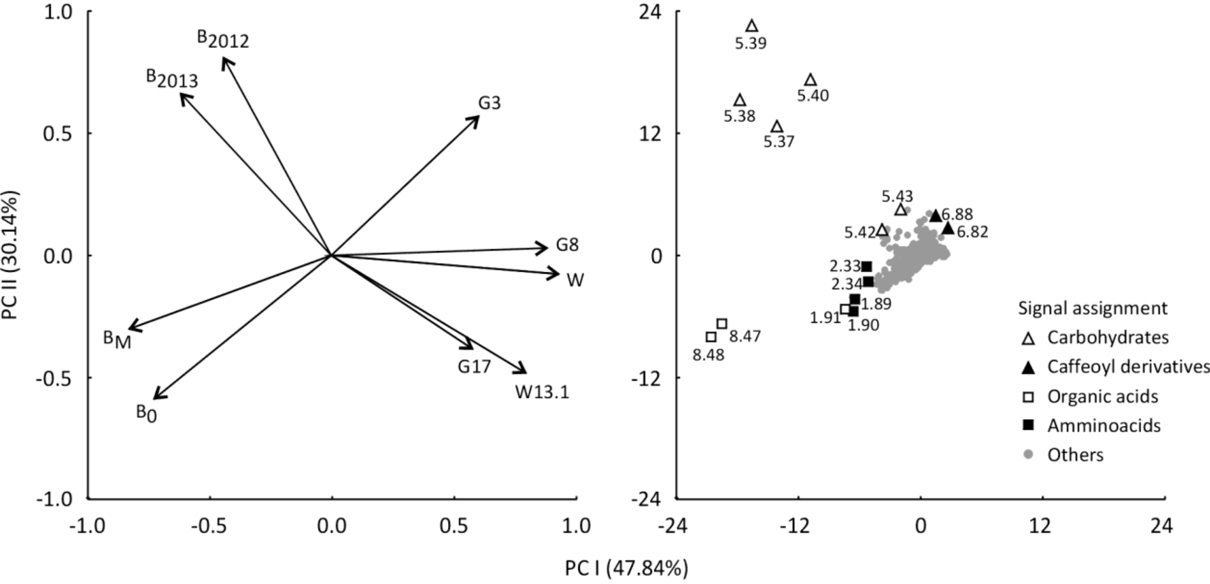

Fig. 5.5 PCA of chia seeds based on ^{1}H NMR spectra in D_2O, excluding the spectral region from 3.20 to 4.10 ppm. Left: vector loadings of chia seeds; Right: factorial scores of resonance intervals of 0.01 ppm, symbolized according to ^{1}H NMR signal assignment. Explained variance of principal components is reported in brackets on the axis labels.

5.3.10 Chemometrics for single metabolites

The ANOVA results showed that the content of all the tested metabolites was significantly different among the populations grown in pots, with the only exception of glucose (Table 5.2). This was linked to the low variability of the three replicated spectra within each chia line. Considering the relative abundance of single metabolites in the samples, post-hoc pair-wise comparisons showed an interesting pattern for different classes of compounds, selectively most or least abundant in the seeds of specific chias (Table 5.2). In particular, the content of all detected amino acids was significantly highest and lowest in the seeds of long-day genotypes G17 and G3, respectively, with the exception of aspartic acid, being most abundant in commercial seeds (B_{2012}, B_{2013}, W) and showing the minimum value in the seeds of long-day

genotype W13.1 (Table 5.2). Interestingly, this genotype showed the highest content of many compounds, such as the carbohydrates sucrose and raffinose, (but not of glucose) and the flavonoids genistein and quercetin, as well as the caffeoyl derivatives caffeic, chlorogenic, and rosmarinic acids (Table 5.2). For the same metabolites, the minimum content was observed in the seeds of long-day genotypes G3 (for sucrose and raffinose) and G8 (for caffeoyl derivatives and genistein), with the exception of quercetin, being most abundant in the commercial black B_{2012} and B_{2013} (Table 5.2). Considering previous reports, Marineli et al. (2014) found that the content of carbohydrates in chia seeds is about 34.57 g/100g. Our results are compatible with this value, given that, limited to glucose, sucrose and raffinose, the total content recorded in our study ranges from 20.8 g/100g in the seeds of W13.1 to 25.5 g/100g in G3 (i.e. 208 to 255‰, Table 5.2). Even though a direct comparison with our data is complicated by the overlapping signals for sugar protons that we observed in the spectral region from 3.40 to 4.10 ppm, an indirect estimate, based on the total integral of this spectral region, produced values, ranging between 60.1% in G17 and 75.8% in G8. In the case of amino acids, Weber et al. (1991) reported that total content of protein in seeds of three different chia populations ranged between 19.0 to 26.5%. Our results are not directly comparable with those previous reports, since our assessment is limited to the total content of specific free amino acids detected from ^{1}H NMR spectra (Table 5.1), rather than to the total protein content. Such value, in our dataset, ranged from 1.56% in G3 to 2.87% in G17 (i.e. 15.6 to 28.7‰, Table 5.2). Differently from other metabolites, maximal and minimal values of the content of single organic acids were unevenly distributed among populations (Table 5.2). In particular, citric acid was most and least abundant in the seeds of long-day genotypes G8 and G3, respectively; seeds of W13.1 showed the highest content of malonic acid and lactic acid, G17 of succinic and formic acids, and B_{2013} of fumaric acid. The lowest content of malonic, lactic, succinic, formic and fumaric acid was recorded in the seeds of B_{2013}, G3, G3, G8 and W13.1 Chias, respectively (Table 5.2). To the best of our knowledge, no direct evidence can be found in the literature for comparison, and this report

represents the first finding of short-chain carboxylic acids in chia seeds. This is important because organic acids besides the key role in the metabolism of cells, including amino acid biosynthesis, energy production and adaptation of the plant to the environment, also participate in the mechanisms used by plants to cope with nutrient lack, metal tolerance and plant-microbe interactions, operating at the root-soil interface (Lòpez-Bucio et al., 2000). Considering differences among seeds in the content of fatty acids (Table 5.2), the long-day genotype G17 showed by far the highest content of MUFA, PUFA, and allylic and diallylic carbons, as well as the highest and lowest abundance of ω3-CH and ω1-CH_3, respectively. Commercial black chias, particularly B_{2012}, showed a different pattern, with highest content of ω1-CH_3 and lowest of ω3-CH, and the highest content of β-CH_2 and γ-CH_2 in B_{2012} and in B_{2013}, respectively. Seed oils from major oilseed crops contain five fatty acids in high abundance (16:0, 18:0, 18:1, 18:2, and 18:3) although many other minor fatty acids are present and may have importance for industrial applications (Liu et al., 2015). Our approach did not allow to determine the length of the fatty acids chain but showed differences in the saturated and omega series of the analyzed material (Table 5.2).

5.3.11 Effects of mineral fertilization

Our chemometric analysis of the content of single metabolites in the seeds of commercial black treated with mineral nitrogen supply, as compared to untreated control, showed that mineral fertilization had a general reducing effect on the content of carbohydrates and flavonoids, positive while the effects on organic acids, polyphenols and fatty acids were highly dependent on the single metabolite considered (Table 5.2). Moreover, the magnitude of such effects was highly variable for single different compounds. In the case of the carbohydrates glucose, sucrose and raffinose, all showing higher mean content in B_0 compared to B_M, the difference due to fertilization was only marginally significant for sucrose, and not significant for glucose

and raffinose (Table 5.2). Interestingly, the content of these three sugars in both B_0 and B_M were much lower than in chias grown in pots, thus indicating a higher production and/or a different partitioning of photosynthates in such conditions. Differences in flavonoid content due to fertilization, although relatively small, were statistically significant in the case of genistein, and marginally significant for quercetin (Table 5.2). Comparison on the total amino acids content could not be done because many signals of amino acids are overlapped by other signals belonging to different classes of metabolites (e.g. flavonoids, phenols and carbohydrates). Those quantified were only a limited number of the total amino acids. However, all amino acids detected in the samples were most abundant in fertilized seeds, but the content increase due to nitrogen mineral supply was statistically significant only for aspartic acid and proline (Table 5.2). Caffeoyl derivatives were evenly distributed between B_0 and B_M seeds, with the exception of chlorogenic acid, showing higher content in un-fertilized samples. Similarly, organic acids showed not significant or marginally significant differences due to fertilization in 5 cases out of 6, the exception being lactic acid, with a higher content in fertilized seeds (Table 5.2). Finally, differences in the content of fatty acids were mostly non-significant, with some exceptions for specific metabolites: MUFA and diallylic carbons were significantly more abundant in un-fertilized samples, while the allylic carbons showed the opposite pattern. Considering differences in chemical composition due to environmental factors and/or experimental treatments, the literature on chia reports an important effect of elevation, with an increase in oil content (Ayerza 2009b) and a decrease in protein level (Ayerza and Coates, 2009a, b, 2011) at high altitutes. Ayerza and Coates (2011) also reported an increase in the levels of α-linolenic and linoleic fatty acid and a decrease in saturated fatty acids, as well as a decrease in the ω-6:ω-3 fatty acid ratio, as elevation increases. Amato et al. (2015) showed a higher concentration of chlorophyll, carotenoids and α-linolenic acid in chia grown in Italy than in commercial black and white chias. The same authors demonstrated a higher concentration of pigments and a lower oxidative stability in chia grown with mineral fertilization. Heuer et al.

(2002) found a reduction on the amount of oil content with an increase in palmitic and linoleic acid content, in chia grown with saline irrigation. Ixtaina et al. (2011) found β-carotene by HPLC analysis. Amato et al. (2015) found carotenes and chlorophyll in oil, as well as tocopherols, with γ-tocopherol as the main component, followed by α- and δ-tocopherol, whereas β-tocopherol was not detected, in accordance with other reports (Ixtaina et al., 2011, Capitani et al., 2012). Finally, according to Amato et al. (2015), nitrogen supply did not affect either the fatty acid composition or oil content of chia seed, which is consistent with our results.

Table 5.2. Relative abundance (‰) of main metabolites detected by ^{1}H NMR analysis. Data refer to mean and standard deviation of 3 replicates. Results of ANOVA (F and p) and post-hoc Tukey's HSD (letters, highest values underlined) testing for single-metabolite differences between chia seeds, as well as t-test for differences between fertilized (B_M) and non-fertilized (B_0) chias, are also reported. Significant ($p < 0.00156$) and marginally significant ($0.00156 < p < 0.05$) p-values in bold.

Metabolite	Chia populations							ANOVA		Effect of mineral N fertilization			
	W13.1	G17	G8	G3	B_{2013}	B_{2012}	W	F	p	B_0	B_M	t	p
Organic acids													
Citric acid (CI)	5.9±0.4 b	6.2±0.3 bc	7.4±0.3 c	3.3±0.2 a	5.6±0.7 b	4.9±0.6 b	5.8±0.6 b	20.5	**<0.0001**	7.7±0.6	8.5±1	1.12	0.3271
Malonic acid (MA)	2.0±0.1 e	1.8±0.2 e	1.3±0.1 bc	1.6±0.1 de	0.7±0.0 a	1.4±0.1 cd	1.0±0.1 b	44.0	**<0.0001**	1.9±0.2	2.8±0.2	5.20	**0.0065**
Lactic acid (LA)	1.7±0.1 d	1.6±0.0 cd	1.6±0.2 cd	0.5±0.1 a	1.3±0.1 bc	1.1±0.0 b	1.6±0.1 cd	37.8	**<0.0001**	1.4±0.1	2.1±0.1	9.01	**0.0008**
Succinic acid (SU)	3.0±0.4 d	3.8±0.3 e	2.6±0.3 bcd	1.6±0.2 a	2.0±0.1 ab	2.2±0.2 abc	2.7±0.2 cd	22.2	**<0.0001**	4.1±0.3	5.1±0.6	2.61	0.0593
Formic acid (FO)	0.8±0.1 b	4.2±0.2 e	0.1±0.0 a	0.2±0.1 a	1.6±0.2 c	2.8±0.3 d	0.2±0.1 a	296.7	**<0.0001**	6.3±0.6	4.2±0.2	-5.66	**0.0048**
Fumaric acid (FU)	0.2±0.1 a	4.5±0.4 cd	2.5±0.1 b	6.3±0.3 de	9.1±1.0 f	8.0±1.2 ef	3.8±0.7 bc	64.1	**<0.0001**	4.9±0.8	3.1±0.2	-3.51	**0.0247**
Free Amino acids													
Alanine (Ala)	4.2±0.4 c	4.0±0.2 c	4.1±0.3 c	1.5±0.2 a	3.6±0.1 c	2.8±0.1 b	3.9±0.2 c	47.7	**<0.0001**	3.6±0.4	4.6±0.5	2.52	0.0654
γ-aminobutyric (GABA)	3.9±0.7 c	4.7±0.3 c	4.0±0.1 bc	2.4±0.1 a	3.0±0.3 ab	3.3±0.2 ab	4.0±0.7 bc	9.5	**0.0003**	3.9±0.4	6.8±0.6	7.14	**0.0020**
Aspartic acid (Asp)	1.9±0.1 a	2.6±0.4 abc	2.0±0.1 ab	2.3±0.3 abc	2.7±0.2 bc	2.9±0.5 c	2.7±0.1 bc	6.1	**0.0025**	2.4±0.1	3.7±0	28.72	**< 0.0001**
Isoleucine (Ile)	1.9±0.2 a	3.2±0.2 c	2.3±0.2 ab	1.8±0.0 a	2.1±0.2 a	2.0±0.3 a	2.8±0.5 bc	11.0	**0.0001**	1.8±0.2	2.5±0.3	3.29	**0.0301**
Leucine (Leu)	0.8±0.1 a	1.2±0.1 b	0.9±0.0 a	0.7±0.1 a	0.7±0.1 a	0.9±0.1 a	1.2±0.1 b	25.8	**<0.0001**	0.5±0	0.8±0.1	7.48	**0.0017**
N-acetylglutamic (NAcGlu)	3.9±0.6 cd	4.8±0.5 d	3.6±0.4 bc	2.4±0.2 a	2.6±0.2 ab	3.2±0.4 abc	3.6±0.3 bc	12.8	**<0.0001**	4.4±0.8	6.8±0.9	3.44	**0.0264**
Proline (Pro)	3.0±0.0 b	3.7±0.3 c	3.1±0.1 b	1.8±0.1 a	2.3±0.2 a	2.3±0.2 a	3.0±0.2 b	38.0	**<0.0001**	3±0.1	5.1±0.4	9.18	**0.0008**
Valine (Val)	3.6±0.5 ab	4.5±0.2 b	4.2±0.3 b	2.7±0.2 a	3.7±0.4 ab	3.5±0.5 ab	4.3±0.5 b	7.6	**0.0009**	3.6±0.5	4.3±0.3	2.20	0.0928
Carbohydrates													
Glucose (Glc)	65±2.3 a	69.3±3.7 a	63.2±8.3 a	72.3±6.1 a	64.5±1.4 a	73.3±6.5 a	72.8±3.1 a	2.2	0.1059	59.5±1.9	47.8±8.1	-2.44	0.0710
Raffinose (Raff)	82.4±8.1 a	84.7±7.2 a	101±8.5 ab	103.8±7.0 b	100.8±2.2 ab	90.8±11.5 ab	91.3±10.8 ab	3.0	**0.0407**	82.2±3.8	75.5±4.7	-1.92	0.1275
Sucrose (Sucr)	59.5±9.0 a	67.7±4.7 ab	64.0±1.3 ab	78.5±5.5 b	67.0±7.0 ab	73.6±5 ab	67.4±4.2 ab	3.6	**0.0228**	53.7±3.6	41.6±3.1	-4.36	**0.0121**
Flavonoids													
Genistein (GE)	1.4±0.2 a	2.2±0.0 bc	3.0±0.2 d	1.9±0.1 ab	2.0±0.2 bc	2.0±0.2 bc	2.4±0.2 c	25.8	**<0.0001**	2.7±0.2	2±0.1	-5.49	**0.0053**
Quercetin (QUE)	1.2±0.1 a	2.2±0.1 bc	2.3±0.4 bc	2.3±0.2 bc	2.7±0.1 c	2.6±0.2 c	2.0±0.1 b	20.4	**<0.0001**	1.6±0.2	1.3±0.1	-2.81	**0.0484**
Caffeoyl derivatives													
Caffeic acid (CA)	2.2±0.2 a	3.6±0.3 b	4.6±0.3 c	3.1±0.2 b	3.1±0.5 ab	3.8±0.3 b	3.3±0.3 b	18.3	**<0.0001**	1.8±0.2	1.6±0.1	-1.39	0.2381
Chlorogenic acid (Chlo)	4.0±0.4 a	5.5±0.4 b	6.9±0.6 c	5.5±0.3 b	4.6±0.6 ab	4.9±0.6 ab	4.6±0.2 ab	11.5	**0.0001**	2.9±0.1	2.1±0.2	-5.70	**0.0047**
Rosmarinic acid (RO)	9.2±0.9 a	12.9±2.0 b	14.0±0.3 b	9.8±1.1 a	9.4±0.6 ab	11.2±0.9 ab	11.4±0.4 ab	9.5	**0.0003**	7.7±0.8	9.3±0.5	2.75	0.0513
Fatty acids													
ω1-CH_3	36±4.2bc	23.5±2.3 a	32.5±3.8 ab	36.5±6.1 bc	30.5±2.5 abc	39±5.3 c	26.8±1.5 ab	5.9	**0.0031**	37.6±3.5	36.5±5.5	-0.31	0.7699
ω3-CH	20.1±1.5bc	27.1±1.9 d	19.3±2.0 abc	19±1.6 bc	15.6±2.7 ab	14.8±1.3 a	23.9±1.9 cd	16.2	**< 0.0001**	21.2±2.2	17.6±1.8	-2.23	0.0901
-(CH_2)n-	90.8±5.3bc	72.6±0.7 a	78.3±6.2 ab	98.2±8.6 c	70.6±6.6 a	96.2±6.6 c	65.8±5.9 a	13.8	**< 0.0001**	73.1±12.6	70.7±6.4	-0.29	0.7880
γ-CH_2	59.2±5.1bc	42.9±3.7 a	53.4±2.0 abc	62.8±0.5 c	48.7±5.7 ab	82.2±9.1 d	40.4±2.8 a	25.5	**< 0.0001**	49.9±5.7	66.2±4.2	3.96	**0.0166**
allylic carbon	11.1±1.5c	41.5±3.1 d	10.4±0.4 bc	5.4±0.4 ab	0.6±0.1 a	7.4±0.5 bc	36.5±3.3 d	229.8	**< 0.0001**	3±0.2	13.3±0.4	36.82	**< 0.0001**
β-CH_2	53.5±2.3cd	43.6±3.2 bc	12±0.3 a	37.2±0.8 b	62.4±9.9 d	43.5±3.3 bc	37.4±3.8 b	37.5	**< 0.0001**	46.5±6.6	53.5±2	1.76	0.1527
diallylic carbon	11.4±1.0b	47.9±7.2 d	1.6±0.2 a	5.6±0.6 ab	3.4±0.4 a	0.5±0.1 a	34.7±1.7 c	129.5	**< 0.0001**	6.7±0.5	0.3±0	-20.25	**0.0000**
glycerol	2.7±0.3bc	2.4±0.2 ab	3.7±0.5 c	3±0.3 bc	3.3±0.5 bc	3.4±0.4 c	1.6±0.1 a	11.0	**0.0001**	3.7±0.4	4.3±0.6	1.41	0.2322
MUFA	8.2±1.1cd	19.9±1.7 e	3.9±0.5 a	8.1±0.3 c	5±0.3 ab	7.1±0.1 bc	10.4±0.6 d	124.2	**< 0.0001**	5.9±0.5	5.2±0.4	-1.86	0.1360
PUFA	13.1±1.8b	51.9±2.7 d	1.8±0.3 a	9.4±0.9 b	0.3±0 a	1.2±0.1 a	46±2 c	665.5	**< 0.0001**	8.2±0.9	1.2±0.1	-14.00	**0.0002**

5.4 Conclusion

The NMR metabolomic study followed by chemometrics of nine chia (*Salvia hispanica* L.) seeds genotypes was performed. Samples analysed included three commercial (two black, and one white), four early flowering (G3, G8, G17, W13.1), and two black genotypes grown with different mineral nitrogen supply. The research aimed at evaluating the chemical composition of the different populations. Results showed that apolar organic extracts were mainly composed of mono- and polyunsaturated fatty acids, such as α-linolenic acid, while polar organic extracts contained glucose, raffinose and sucrose as main metabolites along with caffeoyl derivatives and flavonoids. Several organic acids and free amino acids were also identified in the seed extracts. The nor-diterpenoid, Tashinone I and 15,16 dihydro Tanshinone I were detected in chia seeds for the first time. From the quantitative point of view, the PCA based on proton nuclear magnetic resonance from apolar and aqueous fractions produced opposite results, in terms of differences among the populations. Concerning the apolar fraction, PCA of all spectral signals from $CDCl_3$ extracts showed a certain similarity among the studied seeds. Minor differences among the samples were only recorded in the relative content of these compounds. Concerning the polar fractions, PCA clearly separated the samples showing significant differences for signals related to carbohydrates, which were overall most abundant in commercial black, while commercial white and long-day flowering genotypes showed a lower content of these metabolites. The genotype G3 showed an intermediate pattern. In addition, the analysis of blackseeds, cultivated at different nitrogen supply, showed a decrease in the content of carbohydrates and flavonoids, an increase of aliphatic amino acids and no change in the content of organic acids. The obtained results showed that the combined approach of ^{1}H NMR spectroscopy and multivariate data analysis can provide a detailed metabolomic profiles of biological samples defining the main classes of metabolites both from qualitative and quantitative point of view. Furthermore, the finding of signals characteristics for Tashinone I and 15,16 dihydro Tanshinone I shows that this approach can be used not only to assess major

compounds of the organic extracts, but also to detect metabolites also present in minor amounts. The use of ^{1}H NMR coupled with multivariate data analysis has been widely used to characterize plant metabolomics (e.g. Verpoorte, 2008; Incerti, 2013). However, to the best of our knowledge, this is the first application of such approach to provide a metabolomic fingerprinting of chia seeds.

References

Ahamed Ahmed, M., Ting, I.P., Scora, R.W., 1994. Leaf oil composition of *Salvia hispanica* L. from three geographical areas. J. Essent. Oil Res. 6, 223–228.

Amato, M., Caruso, M.C., Guzzo, F., Galgano, F., Commisso, M., Bochicchio, R., Labella, R., Favati, F., 2015. Nutritional quality of seeds and leaf metabolites of Chia (*Salvia hispanica* L.) from Southern Italy. Eur. Food Res. Technol. 24, 615–625.

Ayerza, R., 1995. Oil content and fatty acid composition of Chia (*Salvia hispanica* L.) from five northwestern locations in Argentina. J. Am. Oil Chem. Soc. 72, 1079–1081.

Ayerza, R., Coates, W., 2004. Protein and oil content, peroxide index and fatty acid composition of Chia (*Salvia hispanica* L.) grown in six tropical and subtropical ecosystems of South America. Trop. Sci. 44, 131–135.

Ayerza, R., Coates, W., 2009a. Some quality components of four Chia (*Salvia hispanica* L.) genotypes grown under tropical coastal desert ecosystem conditions. Asian J. Plant Sci. 8, 301–307.

Ayerza, R., Coates, W., 2009b. Influence of environment on growing period and yield, protein, oil and linolenic content of three Chia (*Salvia hispanica* L.) selections. Ind. Crop Prod. 30, 321–324.

Ayerza, R., Coates, W., 2011. Protein content, oil content and fatty acid profiles as potential criteria to determine the origin of commercially grown Chia (*Salvia hispanica* L.). Ind. Crop Prod. 34:1366–1371.

Ayerza R (2013) Seed composition of two Chia (*Salvia hispanica* L.) genotypes which differ in seed color. Emir J Food Agric 25 (7), 495–500.

Bochicchio, R., Philips, T.D., Lovelli, S., Labella, R., Galgano, F., Di Marsico, A., Perniola, M., Amato, M., 2015a. Innovative Crop Productions for Healthy Food: The Case of Chia (*Salvia hispanica* L.) In: A. Vastola (ed.) The Sustainability of Agro-Food and Natural Resource Systems in the Mediterranean Basin 2015, Springer-Verlag Berlin and Heidelberg pp 15-27.

Bochicchio, R., Rossi, R., Labella, R., Bitella, G., Perniola, M., Amato, M. 2015b. Effect of sowing density and nitrogen top-dress fertilization on growth and yield of Chia (*Salvia hispanica* L.) in a Mediterranean environment. First results. It. J. Agr. 10, 163–166.

Brahmi, N., Scognamiglio, M., Pacifico, S., Mekhoukhe, A., Madani, K. Fiorentino, A., Monaco, P. 2015. ^{1}H NMR based metabolic profiling of eleven Algerian aromatic plants

and evaluation of their antioxidant and cytotoxic properties. Food Res. Intern. 76, 334-341.

Bushway, A.A., Wilson, A.M., Houston, L., Bushway, R.J., 1984. Selected properties of the lipid and protein fractions from Chia seed. J. Food Sci. 49, 555–557.

Cahill, J.P., 2004. Genetic diversity among varieties of Chia (*Salvia hispanica* L.). Genet. Resour. Crop. Evol. 51, 773–781.

Capitani, M.I., Spotorno, V., Nolasco, S.N., Tomás, M.C., 2012. Physicochemical and functional characterization of by-products from Chia (*Salvia hispanica* L.) seeds of Argentina. LWT-Food Sci. Technol. 45, 94–102.

Choi, H.-K., Choi, Y.H., Verbene, M., Lefeber, A.W.M., Erkelens, C., Verpoorte, R., 2004. Metabolic fingerprinting of wild type and transgenic tobacco plants by ^{1}H NMR and multivariate analysis technique. Phytochemistry 65, 857–864.

Choi, Y.H., Sertic, S., Kim, H.K., Wilson, E.G., Michoupoulos, F. Lefeber, A.W.M., Erkelens, C., Prat Kricun, S.D., Verpoorte, R., 2005. Classification of *Ilex* Species Based on Metabolomic

Fingerprinting Using Nuclear Magnetic Resonance and Multivariate Data Analysis. J. Agric. Food Chem. 53, 1237–1245.

Coates, W., Ayerza, R., 1996. Production potential of Chia in northwestern Argentina. Ind. Crop Prod. 5 (3), 229–233

Coates, W., Ayerza, R., 1998. Commercial production of Chia in northwestern Argentina. J. Am. Oil Chem. Soc. 75, 1417–1420.

de Falco, B., Incerti, G., Amato, M., Lanzotti, V., 2015. Artichoke: botanical, agronomical, phytochemical, and pharmacological overview. Phytochem. Rev. 14, 993–1018.

Dai, H., Xiao, C., Liu, H., Tang, H., 2010. Combined NMR and LC-MS Analysis Reveals the Metabonomic Changes in *Salvia miltiorrhiza* Bunge Induced by Water Depletion J. Proteome Res. 9, 1460–1475.

Gentry, H.S., Mittleman, M., McCrohan, P.R., 1990. Introduction of Chia and gum tragacanth in the US. In: Advances in new crops. Proceedings of the first national symposium ‘New crops: research, development, economics, Indianapolis, IN, USA, 23–26 October 1988. Timber, Portland, OR, pp. 252–256.

Hermoso-Diaz, I.A., Velázquez-González, M.A., Lucio-Garcia, M. A., Gonzalez-Rodriguez, J.G., 2014. A Study of *Salvia hispanica* as Green Corrosion Inhibitor for Carbon Steel in Sulfuric Acid. Chem. Sci. Rev. Lett. 3, 685-697

Hernandez-Gomez, J.A., Miranda-Colın, S., 2008. Morphological characterization of Chia (*Salvia hispanica*). Rev. Fitotec. Mex. 31, 105–113.

Heuer, B., Yaniv, Z., Ravina, I., 2002. Effect of late salinization of Chia (*Salvia hispanica*), stock (*Matthiola tricuspidata*) and evening primrose (*Oenothera biennis*) on their oil content and quality. Ind. Crop Prod. 15, 163–167.

Incerti, G., Romano, A., Termolino, P., Lanzotti, V., 2013. Metabolomic fingerprinting using nuclear magnetic resonance and multivariate data analysis as a tool for biodiversity informatics: a case study on the classification of *Rosa x damascena*. Plant Biosys. 147, 947–954.

IUSS Working Group, W.R.B., 2006. World reference base for soil resources. World Soil Resources Report, 103.

Ixtaina, V.Y., Martínez, M.L., Spotorno, V., Mateo, C.M., Maestri, D.M., Diehl, B.W.K., Nolasco. S.M., Tomás, M.C., 2011.Characterization of Chia seed oils obtained by pressing and solvent extraction. J Food Compost Anal. 24, 166–174.

Jamboonsri, W., 2010. Improvement of new oil crops for Kentucky. Doctoral dissertations, University of Kentucky, paper 120. http://uknowledge.uky.edu/gradschool_diss/120.

Jamboonsri, W., Phillips, T.D., Geneve, R.L., Cahill, J.P., Hildebrand, D.F., 2012. Extending the range of an ancient crop, *Salvia hispanica* L. – a new ω3 source. Gen. Res. Crop Evol. 59, 171–178.

Jiang, M., Wang, C., Zhang, Y., Feng, Y., Wanga, Y., Zhua, Y., 2014. Sparse Partial-least-squares Discriminant Analysis for Different Geographical Origins of *Salvia miltiorrhiza* by ^{1}H-NMR-based Metabolomics. Phytochem. Anal., 25, 50 –58.

Kim, H. K., Choi, Y. H., Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. Nature Protocols 5, 536 – 549.

Liu, J., Tjellström, H., McGlewa, K., Shawa, V., Ricea, A., Simpson, J., Kosma D., Maa, W., Yang W., Strawsine, M., Cahoon, E., Durrett, T.P., Ohlrogg J., 2015. Field production, purification and analysis of high-oleic acetyl-triacylglycerols from transgenic *Camelina sativa*. Ind. Crops Prod. 65, 259–268.

Lòpez-Bucio, J., Nieto-Jacobo, M.F., Ramìez-Rodrìguez, V., Herrera-Estrella, L., 2000. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 160, 1–13.

Lu, Y., Foo, L.Y., 2002. Polyphenolics of salvia. Phytochemistry 59, 117–140.

Marineli, R.S., Aguiar Morae, E., Alves Leinquiste, S., Teixeira Godoy, A., Nogueira Eberlin, M., Marostica, M.R., 2014. Chemical characterization and antioxidant potential of Chilean seeds and oil (*Salvia hispanica* L.). LWT– Food Sci. Technol. 59, 1304–1310.

Mattoli, L., Cangi, F., Maidecchi, A., Ghiara, C., Ragazzi, E., Tubaro, M., Stella, L., Tisato, F., Traldi, P., 2006. Metabolomic fingerprinting of plant extracts. J. Mass Spectrom. 41, 1534–1545.

Martínez-Cruz, O., Paredes-López O., 2014. Phytochemical profile and nutraceutical potential of Chia seeds (*Salvia hispanica* L.) by ultra high performance liquid chromatography. J. Chromatogr. A 1346, 43–48.

Miranda-Colin, S., 1978. Evolucion de cultivares nativos de Mexico. Ciencia y Desarrollo 3, 130–131.

Muñoz, L.A., Cobos, A., Diaz, O., Aguilera, J.M., 2013. Chia seed (*Salvia hispanica*): an ancient grain and a new functional food. Food Rev Int 29 (4), 394–408.

Peiretti, P.G., Gai, F., 2009. Fatty acid and nutritive quality of Chia (*Salvia hispanica* L.) seeds and plant during growth. Anim. Feed Sci. Technol. 148, 267–275.

Reyes-Caudillo, E., Tecante, A., Valdivia-Lopez, M.A., 2008. Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican Chia (*Salvia hispanica* L.) seeds. Food Chem. 107, 656–663.

Segura-Campos, M.R., Ciau-Solıs, N., Rosado-Rubio, G., Chel-Guerrero, L., Betancour-Ancona, D., 2014. Physicochemical characterization of Chia (*Salvia hispanica*) seed oil from Yucatan, Mexico. Agric. Sci. 5, 220–226.

Taga, M.S., Miller, E.E., Pratt, D.E., 1984. Chia seeds as a source of natural lipid antioxidants. J Am. Oil Chem. Soc. 61, 928–931.

Verpoorte, R., Choi, Y.H., Kim, H.K., 2007. NMR-based metabolomics at work in phytochemistry. Phytochem. Rev. 6, 3-14.

Weber, C.W., Gentry, H.S., Kohlhepp, E.A., McCrohan, P.R., 1991. The nutritional and chemical evaluation of Chia seeds. Ecol. Food Nutr. 26 (2), 119-125.

Yeboah, S., Owusu Danquah, E., Lamptey, J.N.L., MoChiah, M.B., Lamptey, S., Oteng-Darko, P., Adama, I., Appiah-Kubi, Z., Agyeman, K., 2014. Influence of planting methods and density on performance of Chia (*Salvia hispanica*) and its suitability as an oilseed plant. Agric. Sci. 2, 14–26.

6. Metabolomic Analysis by UAE-GC MS and antioxidant activity of *Salvia hispanica* seeds grown under different irrigation regimes**

6.1 Introduction

Chia (*Salvia hispanica* L.) is an ancient short-day flowering crop from Central America, re-discovered in the1990s, (Gentry et al., 1990; Coates and Ayerza 1996) and thereafter spreading in several areas of the world, at first due to a favorable market placement linked to its nutraceutical properties, and more recently as a source of compounds of cosmetic, medical and industrial interest (Muñoz et al., 2013; Lu and Foo 2002; Hermoso-Diaz et al., 2014). The product of chia is an indehiscent dry fruit, commonly referred to as “seed”, and it is rich in oil and known as one of the richest natural sources of omega 3 fatty acids (Ayerza 1995; Ayerza and Coates 2011). Chia seeds show a high content of protein with a balanced composition in essential amino acids (Ayerza, 2013) and fiber (Capitani et al., 2012). A part of the fiber is located in the outer cells of the fruit and it is extruded at the fruit surface upon hydration (Muñoz et al., 2012), forming a mucilaginous capsule with rheological properties that make it promising for industrial and medical uses: it is highly hygroscopic, viscous and adhesive (Svec et al., 2016). Many antioxidants have been identified in chia seeds and extracted oil and mucilage, and especially phenolic acids and flavonoids, besides poly-unsaturated fatty acids (e.g. Marineli et al., 2014; Amato et al., 2015, Menga et al., 2017). Chia seeds or their products are therefore increasingly proposed not only as food but also as a component for biodegradable film (Muñoz

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et al., 2012; Capitani et al., 2016), thickening agents (Iglesias-Puig and Haros 2013; Felisberto et al., 2015; Coelho and Salas-Mellado 2015, Menga et al., 2017), and for anti-corrosion (Hermoso-Diaz et al., 2014), cosmetic use (Muñoz et al., 2013) and medical uses (Vuksan et al., 2010). A strong variation in chia seeds composition has been reported: for instance, oil content ranges from little over 20 to over 40% (Ayerza 1995, Ixtaina et al., 2011; Ayerza and Coates, 2004; da Silva Marineli et al., 2014; Coelho et al., 2014). Variability has mainly been researched in relation to genotype and environment: Ayerza (2009) reports a range in total content of fat from 25.93 to 33.50% for the same genotype of chia grown in five different environments. The fatty acids profile, and especially the content of α-linolenic acid is also affected by elevation in seeds of this species, even within the same genotype (Ayerza 1995; Ayerza and Coates 2004; Ayerza and Coates 2011; Martínez-Cruz and Paredes-López 2014; Ayerza and Coates 2011), and in general levels of unsaturation of fatty acids are found to increase at cooler temperatures as for other oil seed crops. In an experiment spanning across different countries in America Ayerza (2009) reports a direct relation between elevation an oil content and an inverse relation between elevation and the content of proteins. Ayerza and Coates (2011) suggest that relations of oil and protein content and oil saturation with elevation are strong enough that they could be used to trace the growing environment of chia. Ayerza and Coates (2009) also found differences in protein content for the same genotypes grown in different environments, but could not prove differences among genotypes within a site, except for one variety in one site. In a further study, Ayerza (2013) could not find significant differences between two genotypes of different seed coat color for protein, oil, fiber, amino acids, and antioxidant content. Silva et al., (2016) found that a white and a black seed crop with the same seed yield produced different amount of unsaturated fatty acids: the white seed genotype yielded more linoleic and α-linolenic acids (6.0 and 17.0 kg ha^{-1} respectively) than the black seed one. (4.4 and 16.7 kg ha^{-1} respectively). De Falco et al. (2017) studied the metabolic profile of the seeds of seven chia populations, including commercial and early

flowering mutant genotypes and showed significant differences in the metabolic fingerprinting of the different populations.

Very little information is available about the variation in chia seeds composition with agronomic management: Amato et al. (2015) compared nitrogen fertilization regimes on chia seed composition and found a higher p-Anisidine value, content of phenols and oxidative stability in plots fertilized with organic N only, whereas the addition of mineral nitrogen in topdressing increased free acidity, chlorophyll and carotenoids content. De Falco et al. (2017) reported that the effect of mineral nitrogen supply on chia positively affects the content of aliphatic free amino acids, and negatively that of the main carbohydrates and flavonoids. Heuer et al. (2002) found that salinity of irrigation water decreases the oil content of chia seeds and increased their content of palmitic and linoleic acids.

Irrigation is one of the major agronomical factors conditioning crop yield and composition, and namely that of oilseeds (Flagella et al., 2002). Silva et al. (2016) reported that irrigation did not affect significantly yield in terms of whole seeds and linoleic and α-linolenic acids of chia, in an experiment where very low seed yield was recorded even in a fully irrigated treatment, and commented that their results were affected by the fact that a short-day flowering genotype was used at a latitude higher than optimal. More information is therefore needed on the response of chia to irrigation taking photoperiod sensitivity into account.

We compared the response to irrigation of a traditional short-day flowering commercial chia genotype with a long-day flowering mutant at high latitude aiming at the whole metabolic profile, with the hypothesis that irrigation would affect the fatty acid profile and the production of secondary metabolites found in the polar and non-polar extracts of seeds.

6.2 Materials and methods

6.2.1 Chemicals

Reagents used for the extraction procedure and chemical characterization, methanol anhydrous (99.8%), n-hexane anhydrous (95%), 2,2`-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), derivatizating agents methoxyamine hydrochloride and N-methyl-N-trimethylsilyltrifuoroacetamide (MSTFA) were obtained from Sigma-Aldrich (Dorset, UK). N, O-Bis(trimethylsilyl)trifluoroacetamide, Trimethylchlorosilane (BSTFA with 1% TMCS) was purchased from Supelco Analytical (Bellefonte, PA). Pyridine, sodium carbonate anhydrous, Folin–Ciocalteu's reagent and gallic acid were obtained from Fischer Scientific (Loughborough, UK). Sugars, amino acids, organic acids and polyphenols used for identification and quantification were purchased from Sigma-Aldrich (Dorset, UK).

6.2.2 Plant materials

Black chia (*Salvia hispanica* L.) seeds (B) were obtained from a commercial retailer (Eichenhain– Hofgeismar–DE) and seeds of one long-day flowering mutant genotype (G8), were obtained as described in Jamboonsri et al., (2012) and were kindly supplied through an agreement between the University of Basilicata and the University of Kentucky (US).

6.2.3 Growth conditions

Plants were grown in Basilicata (Southern Italy-Lat. N 40° 51′ 37,59″ Lon. E 15° 38′ 49,43″) on a Luvi-vertic Phaeozem (Iuss working group, 2006), loam soil, in the period June-December 2014. A field factorial randomized block design with three replications was established to test the two genotypes B and G8 with two levels of irrigation: I (irrigation at 100% of ET0) and NI (no irrigation). The crop was sown on June 26, 2014 and irrigation treatments were

differentiated at 51 DAS. Precipitation was 197 mm during the experiment, and the I treatment received 224 mm of irrigation. Seeds of G8 and B were harvested respectively at 132 and 173 DAS.

6.2.4 Extraction procedure of the organic phase

Chia seeds were strained in order to remove extraneous matter as dust and straw. The clean seeds were blended in a laboratory mill (IKA Works MF10, Scotland, UK) in order to obtain a fine powder of the organic material. Subsequently, 15 g of the powder were extracted with 80 ml of n-hexane for 2 h under stirring. The mixture was centrifuged at 3700 rpm for 10 min and the supernatant was immediately stored at -80 °C in dark conditions until further analysis. The pellet was washed twice with 20 ml of n-hexane and then centrifuged at 3700 rpm for 10 min. The supernatant was added to the previous fraction and the leftover pellet was stored overnight in a fume hood in order to remove the excess of n-hexane.

6.2.5 Ultrasound assisted extraction (UAE)

Ten grams of defatted chia seeds were extracted with 100 ml of methanol/water (60:40). Sonication was performed at 20 kHz with 50% of power using a Fischer Scientific Ultrasound (model FB705, 700W, 2000 Park Lane, Pittsburgh, PA) under continuous stirring. The probe was a horn-type (model CL-334), which was kept at constant deep into the mixture using a glass beaker of standard geometry. During the extraction, the temperature was monitored and kept constant (25 °C ± 1) using a thermostatic bath. Five ml of each sample were collected after 2, 20, 40 min and centrifugation was carried out at 2500 rpm for 10 min. The supernatant was stored at -80 ºC until further analysis.

6.2.6 Total polyphenol content

Total polyphenolic content (TPC) was determined by spectrophotometry according to the method described by Singleton and Rossi (1965) with some modifications described below: 125 µl of diluted sample (1:10) were mixed with 500 µl of distilled water and 125 µl of Folin-Ciocalteu reagent. After 6 min, 1.25 ml of sodium carbonate solution at 7.5% were added to the mixture and brought to a final volume of 3 ml with distilled water. The test tubes were then allowed to stand at dark for 90 min at room temperature. The absorbance was read at 760 nm (Thermo Scientific Genesys 10S UV-Vis Spectrophotometer) and TPC was expressed in terms of gallic acid equivalents (GAE/g). A calibration curve ranging from 20 to 200 µg ml^{-1} was used to quantify the TPC content in the seeds extracts. All determinations were performed in triplicate.

6.2.7 Antioxidant activity

The free radical-scavenging activity was determined according to Re et al., 1999 using the reduction of radical cation $ABTS^{\bullet+}$. A mixture of 2.5 ml ABTS 7 mM and 44 µl potassium persulfate 140 mM was prepared and leaved overnight in the dark. The spectrophotometer wavelength was set at 734 nm. The stock solution of ABTS was diluted to 1:80 until a final OD reaches a value between 0.7 and 0.8 nm. 100 µl of diluted sample (1:10) were added to 1 ml of ABTS solution and after 2.5 min its reduction was measured as the percentage of inhibition. Results were expressed in mmol Trolox equivalent antioxidant capacity (TEAC/g) and referred to a calibration curve ranging from 25 to 250 µM. All determinations were performed in triplicate.

6.2.8 Derivatization and GC/MS analysis

In order to obtain volatile and stable compounds, both polar and non-polar extracts were derivatized before the GC/MS analysis. For this purpose, 200 µl of the organic phase were dried under nitrogen stream and a stock solution was prepared. A subsample of 75 µl (500 ppm) was dissolved in 300µl of pyridine/BSTFA + 1% TMCS (1:1) the vials were vortexed, leaved at 25°C for 15 min and analyzed by GC/MS (Shareef et al., 2006). The metabolomic analysis of the polar extract need of two steps processes, starting with oximation to reduce tautomerism of aldehydes and ketones, followed by OH, SH and NH silylation (Gullberg et al., 2004). An aliquot (200 µl) of diluted sample (1:50) was evaporated to dryness in a vacuum centrifuge (Eppendorf Concentrator 5301) and oxymated with 50 µl of methoxyamine hydrochloride (20 mg ml^{-1}) in pyridine at 60°C for 45 min. Samples were then silylated with MSTFA at 60°C for 45 min. Both polar and non-polar extracts were analyzed by gas mass spectrophotometer similarly. One µl of each derivatized sample was injected in a pulsed splitless mode into an Agilent-7820A GC system with 5977E MSD operating in EI mode at 70 eV. The system was equipped with a 30 m x 0.25 mm id fused-silica capillary column with 0.25 µm HP-5MS stationary phase (Agilent technologies, UK). The injection temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1 ml min^{-1}. Separation of non-polar extract was achieved with a temperature program of 80°C for 1 min, then ramped at 10°C/min to 320°C and hold for 1 min. The analysis of the polar compounds was performed under the following temperature program: 2 min of isothermal heating at 70°C, followed by a 10°C/min oven temperature ramp to 320°C, and a final 2 min heating at 320°C. The system was then temperature equilibrated for 1 min at 70°C before injection of the next sample. All spectra were recorded in the mass range 50 to 800 m/z. Both chromatograms and mass spectra were evaluated using the MassHunter Qualitative Analysis B.07.00. Mass spectra of all detected compounds were compared with standard compounds and with spectra in National Institute of Standard and Technologies library NIST MS Search 2.2. Data were processed with the AMDIS software to

deconvolute co-eluting peaks. Artifact peaks, such as peaks due to derivatizing agents, were not considered in the final analysis. Peak areas of multiple peaks belonging to the same compound were summed together. The relative amount of separated metabolites was calculated from Total Ion Chromatography (TIC) by the computerized integrator and with internal standard, malonic acid and 1-oleoyl-rac-glycerol, respectively added to polar and non-polar extract.

6.2.9 Statistical analysis

Relative quantification was done by integrating the peak areas of the chromatographic profiles for each compound and normalizing the data to internal standards. The effect of genotype, irrigation and their interaction on the chemical composition of chia seeds was evaluated through the analysis of variance with the R software (R Development Core Team, 2008).

6.3 Results and discussions

6.3.1 Seeds production and chemical properties

The genotype x irrigation interaction for seed yield is reported in Fig. 6.1 left. Yields of G8 were in the high yielding end of the range of productive data reported for chia worldwide (Bochicchio et al., 2015a) and were significantly higher than those of B. Irrigated plots yielded more than NI but statistical significance of this difference was reached in G8 only. Results confirm first reports of growing traditional genotypes of chia at high latitudes, where short-day flower induction results in late summer flowering and seed ripening occurs in fall-winter and is hampered by low temperatures, therefore yields are low (Bochicchio et al., 2015b; Silva et al., 2016). In case of low yields a lack of significance of irrigation is also reported by Silva et al. (2016). Oil yield (Fig. 6.1 left) was 39.6% on average without significant differences between treatments. This values is in the high range of chia seeds oil content (Bochicchio et al., 2015a).

The TPC of different chia seeds was evaluated using the Folin–Ciocalteu method (Fig. 6.1 right top). The results, expressed as mg GAE/g of defatted chia seed, are in agreement with Amato et al. (2015) and with other previous reports (Reyes-Caudillo et al., 2008; Coelho et al., 2014) but lower compared to data reported by da Silva Marineli et al. (2014). The polyphenol content increased after 40 min of UAE ($p<0.01$). Although in many cases values were lower in irrigated treatments, differences were statistically significant ($p<0.01$) only for the commercial variety at 2 minutes, and sample variability did not allow to reach significance in the other instances. A negative effect of irrigation on TPC for different species is reported in the literature (Dag et al., 2008; Esteban et al., 2001; Patumi et al., 1999 and 2002).

6.3.2 Antioxidant activity

The antioxidant activity of seeds extract was also evaluated and the Fig. 6.1 right bottom shows the TEAC results for Black chia and G8 seeds. The values ranged from 1.317 ± 0.027 to 2.174 ± 0.010 mmol TEAC/g of defatted chia seed, measured after 2 and 40 min, respectively. These results are in agreement with Sargi et al. (2013), but higher than those reported by other authors (Capitani et al., 2012; Vazquez-Ovando et al., 2009). Values were lower in irrigated treatments, but differences were statistically significant ($p<0.01$) only for G8 at 2 minutes. In many plant species, the amount of antioxidants and/or antioxidant activity is shown to increase (e.g. Wu et al., 2017) or remain unaffected (Kyraleou et al., 2016) with reduced water supply. Zhang and Kirkham (1996) show that the degree to which the activities of antioxidant enzymes and the amount of antioxidants change under drought stress is variable with plant species.

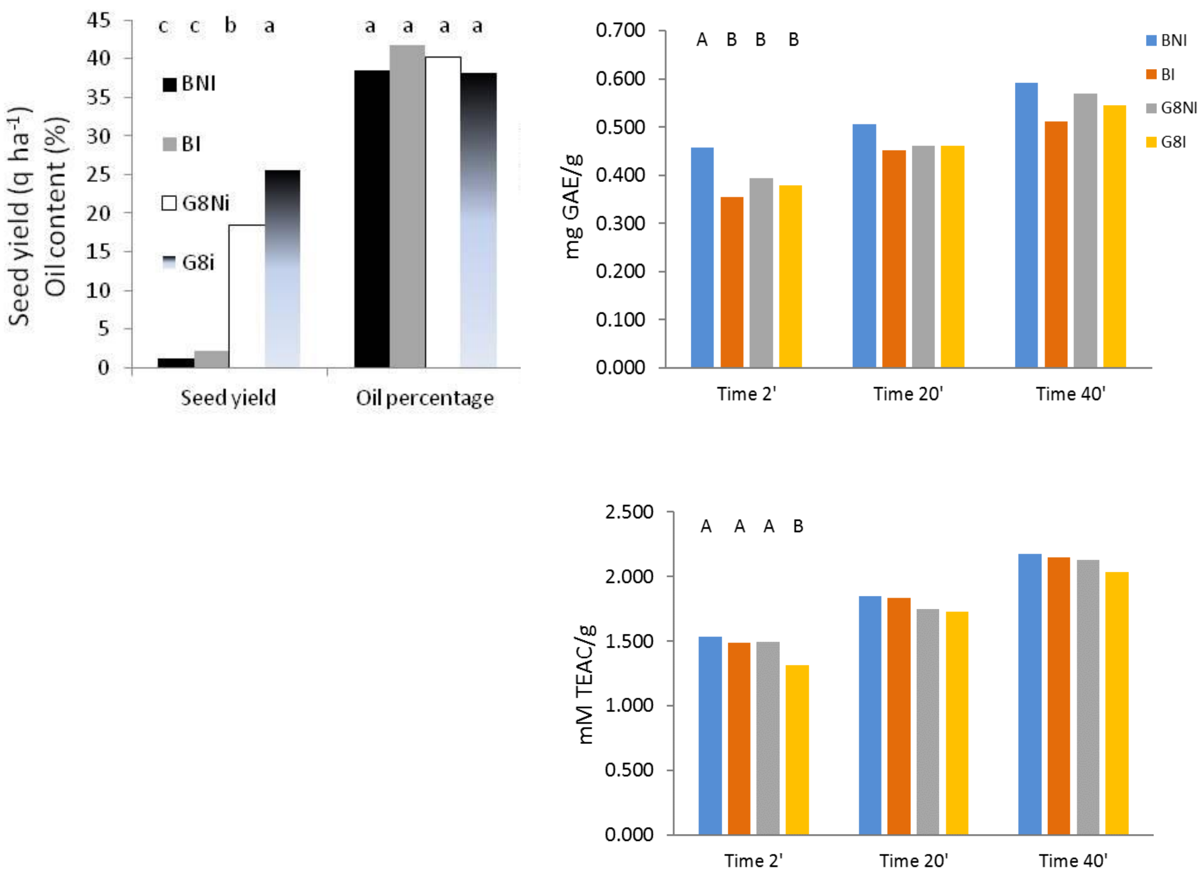

Fig. 6.1 Interaction of genotype x irrigation for yield and chemical properties of chia seeds (*Salvia hispanica* L.). Left: seed yield and oil content. Different lower case letters indicate significant differences ($p<0.05$) at the post-hoc Tukey's test. Right: Total polyphenol content (top) and Antioxidant activity (bottom) of defatted chia seeds. Within each time different upper case letters indicate highly significant differences ($p<0.01$) at the post-hoc Tukey's test.

6.3.3 Metabolite profile

A whole metabolome profile of two different genotypes (G8 and black chia) of *Salvia hispanica* L. seeds was evaluated by GC-MS analysis. Metabolomic fingerprinting of both genotypes supplemented with irrigation was also studied and compared with untreated samples. Each sample was analyzed in triplicate and a total of 34 metabolites, including carbohydrates, amino acids, organic acids and fatty acids, were identified by comparing the GC-MS data with commercial mass spectral libraries, literature data and reference compounds, run under the same experimental conditions. The auto-sampler in GC instrument was used to minimize the effect

of variation during the injections. All compounds of polar and non-polar extract with their respective retention times and *m/z* values are listed in Table 6.1, and representative total ion chromatograms (TIC) for both fractions are reported in Fig. 6.2.

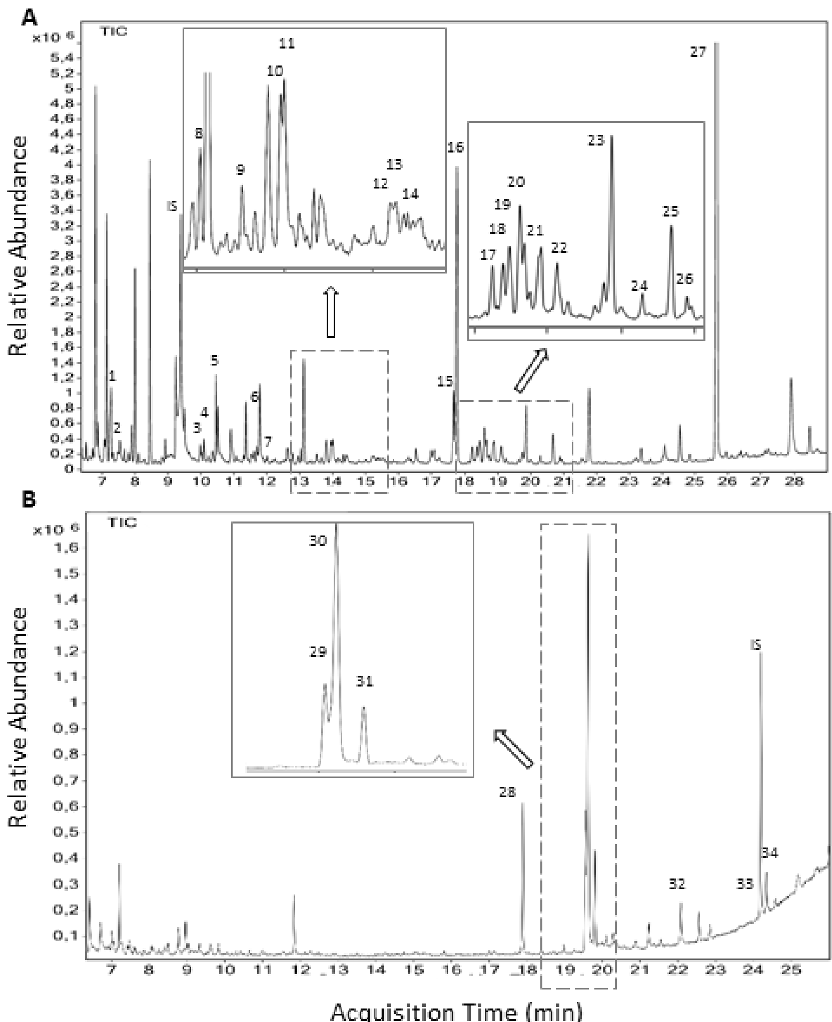

Fig. 6.2 Representative TIC of the polar (A) and non-polar (B) fractions of BNI seeds extracts. Peaks correspond to numbering of compounds in Table 6.1

6.3.4 Apolar phase

The main fatty acids detected in chia seeds were linolenic acid (18:3), linoleic acid (18:2), stearic acid (18:0), palmitic acid (16:0) and oleic acid (18:1), determined as TMS derivatives. These compounds were previously detected in other reports (Amato et al., 2015; da Silva

Marineli et al., 2014; Peiretti et al., 2009). A preliminary analysis of chromatograms showed C18:3 as the most abundant fatty acid in all samples. On the contrary, 10-heptadecenoic acid (17:1), also reported by Gören et al., 2006 in three species of Turkish Salvia, was detected in slightest amount. MS spectrum of saturated fatty acids trimethylsilyl esters such as C16:0 and C18:0 have a base peak at m/z 313 and 341 respectively, which represents the loss of methyl group from TMS ester group, while m/z 132 represents the McLafferty rearrangement ion. In the MS spectrum of monounsaturated and polyunsaturated TMS fatty acids, such as C18:1, C18:2 and C18:3 characteristic peaks for each of them were detected at m/z 339, 337 and 335, respectively. Also in these spectra, a base peak at m/z 73, due to TMS was always detected. As shown in Fig. 6.3, the most abundant fatty acid is α-linolenic acid (C18:3) in all samples, and this is in agreement with those of other reports (de Falco et al., 2017; Amato et al., 2015; da Silva Marineli et al., 2014; Coelho et al., 2014). Analysis of variance on the non-polar fraction (Table 6.2) shows that the main effects of genotype and irrigation were significant in many cases. In particular, the total amount of fatty acids was found higher in G8 than in B, except for C17:1 which was higher in B, and oleic acid (C18:1) and glycerol monostearate (GMS) which were not significantly different between genotypes. Although irrigation treatment affected the fatty acids composition of both genotypes of chia seeds, an interaction was significant in C18:1 where values of the irrigated treatment were higher than NI only for G8, and in GMS where I<NI in G8 and I>NI in B. Silva et al. (2016) reported that irrigation did not affect significantly the content of linoleic and α-linolenic acids of chia, but their experiment tested less extreme levels of irrigation, ranging between 40% and 100% of ET0, whereas in our case 100% of ET0 is compared with rainfed conditions. In other species irrigation is reported to affect fatty acids composition but results are often contrasting. Erdemoglu et al. (2003) found a decrease in the content linoleic and oleic acids with irrigation in sunflower seed oil. Sezen et al. (2011) found an increase in linoleic, palmitic and stearic acid concentrations with irrigation. Bellaloui et al.

(2015) report that irrigation affects soybean oil composition differently according to the degree and stage of differentiation of water treatments.

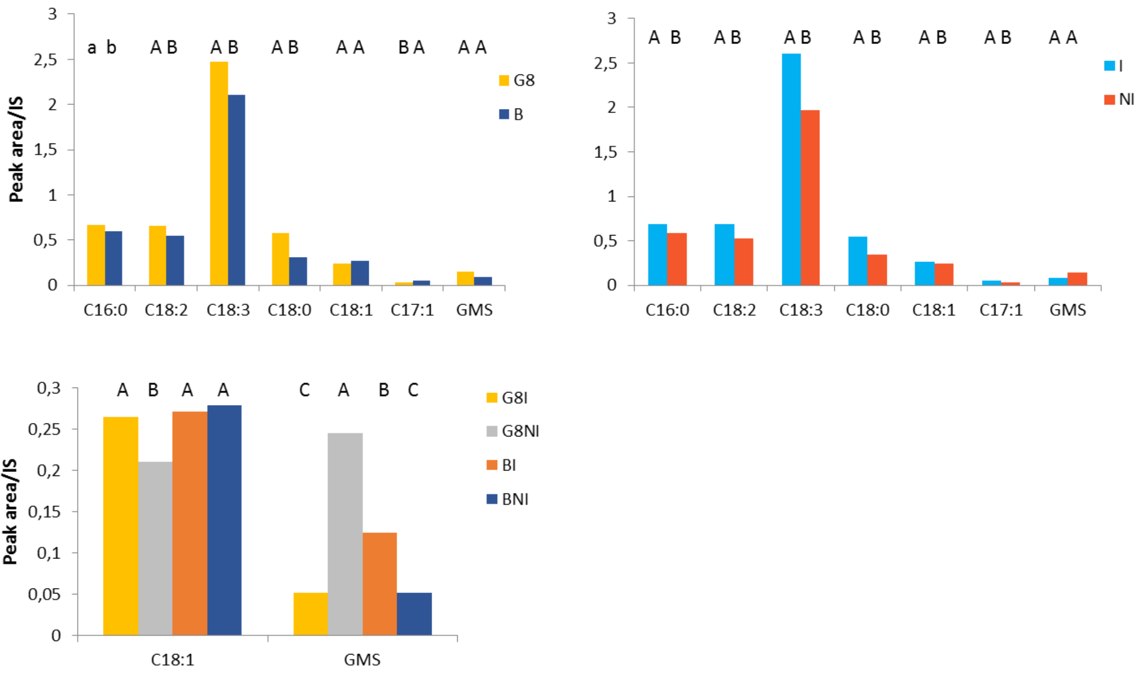

Fig. 6.3 Metabolites belonging to different classes of compounds of non-polar extract of chia seeds detected by GC-MS analysis top left: main effects of genotype; top right: main effect of irrigation; bottom left: interaction of genotype x irrigation. Within each compound different upper case letters indicate highly significant differences ($p<0.01$) and different lower case letters indicate significant differences ($p<0.05$) at the analysis of variance for main effects and at the post-hoc Tukey's test for the interaction.

The percentages of oleic and linoleic acid in plant fats have a reverse relationship, and in chia this has been found by Ayerza (2009). This is linked to the dynamics of oleate desaturases with an increase of desaturation in cold conditions and therefore a decrease of the oleic/linoleic acids ratio in cooler environmental temperatures (Aparicio et al., 1994). In our data, although the amount of many fatty acids increases in response to irrigation, the response is proportionally lower in oleic acid, and the oleic/linoleic ratio decreases from 47.4 in the rainfed samples to 39.6 in the irrigated treatments. Flagella et al. (2002) observed a decrease in the oleic/linoleic

acid ratio in sunflower in response to irrigation, and suggested that a possible thermal effect of irrigation may have affected the activity of oleate desaturase.

6.3.5 Polar phase

In the aqueous extracts sugars are the principal class of compounds and the disaccharide sucrose (m/z 361) represents the major component followed by methyl galactose as the major monosaccharide component (Table 6.1). Other sugars identified were glucose, galactose, fructose, mannitol and glucuronic acids. In particular, TMS derivatives of monosaccharides such as glucose and galactose showed a very similar GC-MS profiles, due to their stereoisomery, with characteristic ions observed at m/z 147, 205, 319 and 364. The final identification of these compounds was achieved by comparing their elution order with literature data (Gómez-González et al., 2010) and by injection of a standard samples. Other compounds detected were the polyphenol caffeic acid, the polyol myo-inositol and a series of carboxylic acids and amino acids (Table 6.1). These data are in agreement with assignments obtained by de Falco et al. (2017) on the metabolomic analysis of G8 and black chia seeds by NMR and chemometrics. A base peak at m/z 73, typical of silylated compounds, was always detected in the chromatograms due to [$(CH_3)_3Si$] group. In the polar fraction (Fig. 6.4) sucrose (Sucr) and methyl-galactoside (mGal) are the most abundant sugars present in all samples, while within organic acids, lactic acid (LA) and citric acid (CI) have the highest value, following by quinic acid (QUI). Caffeic acid (CA) did not exhibit variation within genotype, but even if statistically not significant, its level is higher in black chia than G8. Values were more variable than those of the apolar phase, and statistical significance of the differences between genotype and irrigation treatments was not reached for many compounds (Fig. 6.4 top left). A genotype effect was significant for some compounds (Fig. 6.4 top right): G8 showed a significantly higher amount of lactic acid (LA), benzoic acid (BE), serine (Ser) and aspartic acid (Asp), while B

showed a significantly higher amount of glucuronic acid (GLUC), arabitol (Arab) and mannitol (Man). Irrigation main effects were not significant but interactions were found for some compounds (Fig. 6.4 bottom left): irrigated samples showed significantly higher values of LA and glycine (Gly) in G8 only, and significantly lower values of Asp and phenylalanine (Phe) in B only.

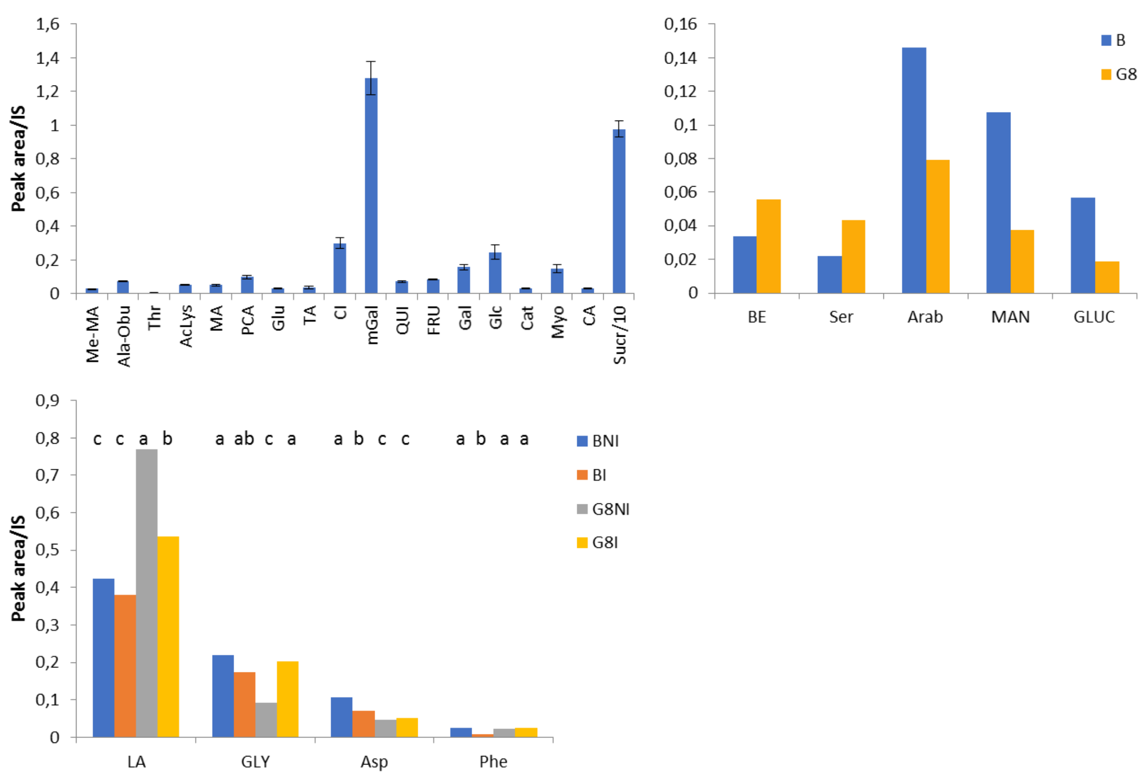

Fig. 6.4 Metabolites belonging to different classes of compounds of non-polar extract of chia seeds detected by GC-MS analysis top left: overall average for compounds not significantly different between treatments; top right: main effect of genotype for compounds significantly different between G8 and B; bottom left: interaction of genotype x irrigation; within each compound different lower case letters indicate significant differences ($p<0.05$) at the post-hoc Tukey's test for the interaction.

Table 6.1 Polar and non-polar metabolites assigned in chia seeds by GC-MS

Peak	Detected metabolites	Abbreviation	RT (min)	Molecular formula	*m/z*
Polar					
1	Lactic Acid	LA	7.25	$C_9H_{22}O_3Si_2$	219, 191, 147, 133, 117, 73, 45
2	Methyl 2-ethyl malonate	Me-MA	7.32	$C_9H_{18}O_4Si$	175, 89, 73
3	L-Alanine, N-methyl-N-(trifluoroacetyl)-, butyl ester	Ala, Nm-Tfa-Obu	9.99	$C_{10}H_{16}F_3NO_3$	154, 110
4	Benzoic Acid	BE	10.03	$C_{10}H_{14}O_2Si$	194, 179, 135, 105, 77
5	Glycerol	GLY	10.46	$C_{12}H_{32}O_3Si_3$	218, 205, 147, 117, 89, 73, 45
6	L-serine	Ser	11.74	$C_{12}H_{31}NO_3Si_3$	218, 204, 147, 100, 73
7	L-Threonine	Thr	12.14	$C_{13}H_{33}NO_3Si_3$	291, 218, 147, 117, 73
8	N-α-Acetyl-L-Lysine	AcLys	13.04	$C_{17}H_{40}N_2O_3Si_3$	404, 287, 73
9	Malic acid	MA	13.52	$C_{13}H_{30}O_5Si_3$	245, 233, 147, 133, 73
10	L-Aspartic acid	Asp	13.97	$C_{13}H_{31}NO_4Si_3$	232, 218, 147, 100, 73
11	L-5-Oxoproline	PCA	14.00	$C_{11}H_{23}NO_3Si_2$	258, 230, 156, 133, 73, 45
12	L-Glutamic acid	Glu	15.21	$C_{14}H_{33}NO_4Si_3$	246, 147, 128, 73
13	Phenylalanine	Phe	15.35	$C_{15}H_{27}NO_2Si_2$	218, 192, 147, 100, 73
14	Tartaric acid	TA	15.54	$C_{28}H_{62}O_6Si_4$	549, 417, 389, 147, 73
15	Citric acid	CI	17.69	$C_{18}H_{40}O_7Si_4$	273, 147, 73, 45
16	Methyl galactoside	mGal	17.77	$C_{19}H_{46}O_6Si_4$	243, 217, 204, 133, 73
17	Quininic acid	QUI	18.24	$C_{22}H_{52}O_6Si_5$	345, 255, 191, 147, 73
18	D-Fructose MEOX	FRU	18.37	$C_{22}H_{55}NO_6Si_5$	217, 307
19	Arabitol	Arab	18.48	$C_{20}H_{52}O_5Si_5$	307, 277, 217, 189, 147, 103, 73
20	D-Galactose MEOX	Gal	18.61	$C_{22}H_{55}NO_6Si_5$	319, 205, 147, 103, 73
21	D-Glucose MEOX	Glc	18.67	$C_{22}H_{55}NO_6Si_5$	364, 319, 205, 147, 73
22	D-Mannitol	MAN	19.04	$C_{24}H_{62}O_6Si_6$	421, 345, 319, 205, 147, 103, 73
23	D-Gluconic acid	GLUC	19.77	$C_{24}H_{60}O_7Si_6$	333, 292, 205, 147, 103, 73
24	Trimethylsilyl catechollactate tris(trimethylsilyl) ether	Cat	20.30	$C_{21}H_{42}O_5Si_4$	396, 267, 179, 147, 73
25	Myo-Inositol	Myo	20.69	$C_{24}H_{60}O_6Si_6$	305, 217, 147, 129, 73
26	Caffeic acid	CA	20.91	$C_{18}H_{32}O_4Si_3$	396, 381, 219, 191, 73
27	Sucrose	Sucr	25.66	$C_{36}H_{86}O_{11}Si_8$	437, 361, 319, 271, 217, 147, 103, 73
Apolar					
28	Palmitic Acid	C16:0	17.88	$C_{19}H_{40}O_2Si$	328, 313, 145, 117, 73
29	Linoleic acid	C18:2	19.54	$C_{21}H_{40}O_2Si$	337, 129, 95, 75, 73
30	α-Linolenic acid	C18:3	19.61	$C_{21}H_{38}O_2Si$	335, 129, 95, 75, 73
31	Stearic acid	C18:0	19.80	$C_{21}H_{44}O_2Si$	356, 341, 132, 117, 73
32	Oleic acid	C18:1	22.07	$C_{21}H_{42}O_2Si$	354, 339, 129, 117, 73
33	10-Heptadecenoic acid	C17:1	24.05	$C_{20}H_{40}O_2Si$	340, 325, 145, 129, 117, 73
34	Glycerol monostearate	GMS	24.34	$C_{27}H_{58}O_4Si_2$	487, 399, 147, 73

Table 6.2 Analysis of variance on polar and non-polar fraction to evaluate the effect of genotype and irrigation on chia seeds

Compounds	Genotype	Irrigation	Genotype x Irrigation
Polar			
LA	p<0.05	n.s.	p<0.05
Me-MA	n.s.	n.s.	n.s.
Ala-Obu	n.s.	n.s.	n.s.
BE	p<0.05	n.s.	n.s.
GLY	n.s.	n.s.	p<0.05
Ser	p<0.05	n.s.	n.s.
Thr	n.s.	n.s.	n.s.
AcLys	n.s.	n.s.	n.s.
MA	n.s.	n.s.	n.s.
Asp	p<0.01	n.s.	p<0.05
PCA	n.s.	n.s.	n.s.
Glu	n.s.	n.s.	n.s.
Phe	n.s.	n.s.	p<0.05
TA	n.s.	n.s.	n.s.
CI	n.s.	n.s.	n.s.
mGal	n.s.	n.s.	n.s.
QUI	n.s.	n.s.	n.s.
FRU	n.s.	n.s.	n.s.
Arab	p<0.05	n.s.	n.s.
Gal	n.s.	n.s.	n.s.
Glc	n.s.	n.s.	n.s.
MAN	p<0.01	n.s.	n.s.
GLUC	p<0.01	n.s.	n.s.
Cat	n.s.	n.s.	n.s.
Myo	n.s.	n.s.	n.s.
CA	n.s.	n.s.	n.s.
Sucr	n.s.	n.s.	n.s.
Apolar			
C16:0	P<0.05	P<0.001	n.s.
C18:2	P<0.01	P<0.0001	n.s.
C18:3	P<0.01	P<0.0001	n.s.
C18:0	P<0.01	P<0.0001	n.s.
C18:1	n.s.	P<0.0001	P<0.01
C17:1	P<0.01	P<0.0001	n.s.
GMS	n.s.	n.s.	P<0.01

6.4 Conclusion

This work provides a high-throughput analysis of metabolomic fingerprinting including total polyphenolic content (TPC) and antioxidant activity (TEAC), on commercial black chia and early flowering G8 seeds. The analytical approach performed by UAE GC-MS allowed to detect and quantify a high number of metabolites.

The aim of the paper was to evaluate the difference in organic compounds between a wild plant, black chia, and the recently developed mutant G8. The species were grown at different level of irrigation to evaluate the effect of water supply on the metabolite content. Results showed an increase of TPC and antioxidant activity (expressed as TEAC) in all samples after 40 minutes of UAE. On the contrary, a decrease of TPC and TEAC levels was observed after irrigation treatments. Concerning the apolar phase, quantitative analysis showed a higher yield and content of many fatty acids. including ω-3 (α-linolenic), in the early flowering G8 mutant, with a decrease of the ratio of oleic/linoleic acids. Concerning the polar fraction, sugars were found as main metabolites with sucrose and methyl galactose as the major component. The genotype effect has more influence on the aqueous extract than the irrigation treatment. G8 showed a significantly higher amount of some organic acids and amino acids, such us LA, BE, Ser and Asp, while GLUC, Arab and Mann are mainly present in the wild-type.

The obtained results showed how Gas Chromatography-Mass Spectrometry can provide a detailed metabolic profile of chia seeds extracted with ultrasounds. Furthermore, this study highlighted for the first time the effects of irrigation on a late flowering and an early flowering mutant chia genotypes.

References

Amato, M., Caruso, M.C., Guzzo, F., Galgano, F., Commisso, M., Bochicchio, R., Labella, R., Favati, F., 2015. Nutritional quality of seeds and leaf metabolites of Chia (*Salvia hispanica* L.) from Southern Italy. Eur. Food Res. Technol. 24, 615–625.

Aparicio, R., Ferreiro, L., Alonso, V., 1994. Effect of climate on the chemical composition of virgin olive oil. Anal. Chim. Acta 292,235-241.

Ayerza, R., 1995. Oil content and fatty acid composition of Chia (*Salvia hispanica* L.) from five northwestern locations in Argentina. J. Am. Oil Chem. Soc. 72,1079–1081.

Ayerza, R., 2009. Chia (*Salvia hispanica* L.) as affected by environmental factors. J. Oleo Sci. 58,347–354.

Ayerza, R., 2013. Seed composition of two Chia (*Salvia hispanica* L.) genotypes which differ in seed color. EJFA. 25, 495.

Ayerza, R., Coates, W., 2004. Protein and oil content, peroxide index and fatty acid composition of Chia (*Salvia hispanica* L.) grown in six tropical and subtropical ecosystems of South America Trop. Sci. 44,131–135.

Ayerza, R., Coates, W., 2009. Influence of environment on growing period and yield, protein, oil and linolenic content of three Chia (*Salvia hispanica* L.) selections. Ind. Crops Prod. 30,321–324.

Ayerza, R., Coates, W., 2011. Protein content, oil content and fatty acid profiles as potential criteria to determine the origin of commercially grown Chia (*Salvia hispanica* L.). Ind. Crops Prod. 34,1366–1371.

Bellaloui, N., Bruns, H.A., Abbas, H.K., Mengistu, A., Fisher, D.K., Reddy, K.N. 2015. Agricultural practices altered soybean seed protein, oil, fatty acids, sugars, and minerals in the Midsouth USA. Frontiers in plant science, 6, 31.

Bochicchio R, Philips TD, Lovelli S, Labella R, Galgano F, Di Marsico A, Perniola M, Amato M. 2015a. Innovative Crop Productions for Healthy Food: The Case of Chia (*Salvia hispanica* L.) In: A. Vastola (ed.) The Sustainability of Agro-Food and Natural Resource Systems in the Mediterranean Basin 2015, Springer-Verlag Berlin and Heidelberg pp 15-27.

Bochicchio, R., Rossi, R., Labella, R., Bitella, G., Perniola, M., Amato, M. 2015b. Effect of sowing density and nitrogen top-dress fertilisation on growth and yield of Chia (*Salvia hispanica* L.) in a Mediterranean environment: first results. Ital J Agron, 10, 163-166.

Capitani, M.I., Matus-Basto, A., Ruiz-Ruiz, J.C., Santiago-García, J.L., Betancur-Ancona, D.A., Nolasco, S.M., Tomás, M.C., Segura-Campos, M.R., 2016. Characterization of Biodegradable Films Based on *Salvia Hispanica* L. Protein and Mucilage. Food Bioprocess Technol. 9,1276 1286.

Capitani, M.I., Spotorno, V., Nolasco, S.N., Tomás, M.C., 2012. Physicochemical and functional characterization of by-products from Chia (*Salvia hispanica* L.) seeds of Argentina. LWT-Food Sci. Technol. 45, 94–102.

Coates, W., Ayerza, R., 1996. Production potential of Chia in northwestern Argentina. Ind. Crop Prod. 5 (3), 229–233

Coelho, S.M., de las Mercedes Salas-Mellado, M. 2014. Chemical characterization of Chia (*Salvia hispanica* L.) for use in food product. J. Food Nutr. Res. 2,263–269.

Dag, A., Alon, B.-G., Uri, Y., Loai, B., Yogev, N., 2008. The effect of irrigation level and harvest mechanization on virgin olive oil quality in a traditional rain-fed 'Souri' olive orchard converted to irrigation. J Sci Food Agric 88,1524–1528.

da Silva Marineli, R.S., Aguiar Morae, E., Alves Leinquiste, S., Teixeira Godoy, A., Nogueira Eberlin, M., Marostica, M.R., 2014. Chemical characterization and antioxidant potential of Chilean seeds and oil (*Salvia hispanica* L.). LWT– Food Sci. Technol. 59, 1304–1310.

de Falco, B., Incerti, G., Bochicchio, R., Phillips, T., Amato, M., Lanzotti, V. 2016. Metabolomic analysis of *Salvia hispanica* seeds using NMR spectroscopy and multivariate data analysis. Ind. Crop Prod. 99, 86-96.

Erdemoglu, N., Kusmenoglu, S., & Yenice, N., 2003. Effect of irrigation on the oil content and fatty acid composition of some sunflower seeds. Chem. Nat. Compd. 39,1-4.

Esteban, M.A., Villanueva, M.J., Lissarrague, J.R. 2001. Effect of irrigation on changes in the anthocyanin composition of the skin of cv Tempranillo (*Vitis vinifera* L) grape berries during ripening. J Sci Food Agric 81,409–420.

Felisberto, M.H.F., Galvão, M.T.E.L., Picone, C.S.F., Cunha, R.L., Pollonio, M.A.R. 2015. Effect of prebiotic ingredients on the rheological properties and microstructure of reduced-sodium and low-fat meat emulsions. LWT-Food Sci Technol 60, 148-155.

Flagella, Z., Rotunno, T., Tarantino, E., Di Caterina, R., De Caro, A., 2002. Changes in seed yield and oil fatty acid composition of high oleic sunflower (*Helianthus annuus* L) hybrids in relation to sowing date and water regime. European J. Agron. 17,221–30.

Gentry, H.S., Mittleman, M., McCrohan, P.R., 1990. Introduction of Chia and gum tragacanth in the US. In: Advances in new crops. Proceedings of the first national symposium 'New

crops: research, development, economics, Indianapolis, IN, USA, 23–26 October 1988. Timber, Portland, OR, pp. 252–256.

Gómez-González, S., Ruiz-Jiménez, J., Priego-Capote, F., Luque de Castro, M.D. 2010. Qualitative and Quantitative Sugar Profiling in Olive Fruits, Leaves, and Stems by Gas Chromatography− Tandem Mass Spectrometry (GC-MS/MS) after Ultrasound-Assisted Leaching. J Agr Food Chem58, 12292-12299.

Gören, A.C., Kiliç, T., Dirmenci, T., Bilsel, G. 2006. Chemotaxonomic evaluation of Turkish species of Salvia: Fatty acid compositions of seed oils. Biochem syst ecol 34, 160-164.

Gullberg, J., Jonsson, P., Nordstrom, A., Sjostrom, M., Moritz, T. 2004. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/mass spectrometry. Analytic. Biochem. 331, 283–295.

IUSS Working Group, W.R.B., 2006. World reference base for soil resources. World Soil Resources Report, 103.

Hermoso-Diaz, I.A., Velázquez-González, M.A., Lucio-Garcia, M. A., Gonzalez-Rodriguez, J.G., 2014. A Study of *Salvia hispanica* as Green Corrosion Inhibitor for Carbon Steel in Sulfuric Acid. Chem. Sci. Rev. Lett. 3, 685-697

Iglesias-Puig, E., Haros, M. 2013. Evaluation of performance of dough and bread incorporating Chia (*Salvia hispanica* L.). Euro Food Res Technol 237, 865-874

Ixtaina, V.Y., Martínez, M.L., Spotorno, V., Mateo, C.M., Maestri, D.M., Diehl, B.W.K., Nolasco, S.M., Tomás, M.C., 2011. Characterization of Chia seed oils obtained by pressing and solvent extraction. J. Food Compost. Anal. 24,166–174.

Lu, Y., Foo, L.Y., 2002. Polyphenolics of salvia. Phytochemistry 59, 117–140.

Jamboonsri, W., Phillips, T.D., Geneve, R.L., Cahill, J.P., Hildebrand, D.F., 2012. Extending the range of an ancient crop, *Salvia hispanica* L. – a new ω3 source. Gen. Res. Crop Evol. 59, 171–178.

Kyraleou, M., Koundouras, S., Kallithraka, S., Theodorou, N., Proxenia, N., Kotseridis, Y., 2016. Effect of irrigation regime on anthocyanin content and antioxidant activity of *Vitis vinifera* L. cv. Syrah grapes under semiarid conditions. J. Sci. Food Agric. 96,988-96.

Martínez-Cruz, O., Paredes-López, O., 2014. Phytochemical profile and nutraceutical potential of Chia seeds (*Salvia hispanica* L.) by ultrahigh performance liquid chromatography. J. Chromatogr. A. 1346,43–48.

Menga, V., Amato, M., Phillips, T. D., Angelino, D., Morreale, F., Fares, C. 2017. Gluten-free pasta incorporating Chia (*Salvia hispanica* L.) ss thickening agent: an approach to

naturally improve the nutritional profile and the in vitro carbohydrate digestibility. Food Chem. 221, 1954-1961.

Muñoz LA, Cobos A, Diaz O, Aguilera JM (2012) Chia seeds: Microstructure, mucilage extraction and hydration. J Food Eng 108:216-224

Muñoz, L.A., Cobos, A., Diaz, O., Aguilera, J.M., 2013. Chia seed (*Salvia hispanica*): an ancient grain and a new functional food. Food Rev Int 29 (4), 394–408.

Patumi, M., d'Andria, R., Fontanazza, G., Morelli, G., Giorio, P., Sorrentino, G., 1999. Yield and oil quality of intensively trained trees of three cultivars of olive (*Olea europaea* L.) under different irrigation regimes. J. Hortic. Sci. Biotechnol. 74, 729–737.

Patumi, M., d'Andria, R., Marsilio, V., Fontanazza, G., Morelli, G., Lanza, B., 2002. Olive and olive oil quality after intensive monocone olive growing (*Olea europaea* L. cv. Kalamata) in different irrigation regimes. Food Chem. 77, 27–34.

Peiretti, P.G., Gai, F. 2009. Fatty acid and nutritive quality of Chia (*Salvia hispanica* L.) seeds and plant during growth. Animal Feed Science and Technology, 148, 267-275.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26, 1231–1237.

Reyes-Caudillo, E., Tecante, A., Valdivia-López, M.A., 2008. Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican Chia (*Salvia hispanica* L.) seeds. Food Chem. 107, 656–663.

Sargi, S. C., Silva, B. C., Santos, H. M. C., Montanher, P. F., Boeing, J. S., Santos Junior, O.O., Souza, N.E., Visentainer, J.V., 2013. Antioxidant capacity and chemical composition in seeds rich in omega-3: Chia, flax, and perilla. Food Sci. Technol. (Campinas), 33, 541–548.

Sezen, S.M., Yazar, A., Kapur, B., Tekin, S., 2011. Comparison of drip and sprinkler irrigation strategies on sunflower seed and oil yield and quality under Mediterranean climatic conditions. Agric. Water Manage. 98,1153-1161.

Shareef, A., Angove, M.J., Wells, J.D., 2006. Optimization of Silylation using N-methyl-N-(trimethylsilyl)-trifluoroacetamide, N,O-bis-(trimethylsilyl)- trifluoroacetamide and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide for the determination of the estrogens estrone and 17α-ethinylestradiol by gas chromatography–mass spectrometry. J. Chromatogr. A 1108,121–8.

Silva, H., Garrido, M., Baginsky, C., Valenzuela, A., Morales, L., Valenzuela, C., Pavez, S., Alister, S., 2016. Effect of water availability on growth, water use efficiency and omega 3

(ALA) content in two phenotypes of Chia (*Salvia hispanica* L.) established in the arid Mediterranean zone of Chile. Agric. Water Manage. 173, 67–75.

Singleton, V. L., Rossi, J.A., 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid. Am. J. Enol. Vitic. 16, 144-158.

Švec, I., Hrušková, M., & Jurinová, I. 2016. Pasting characteristics of wheat-Chia blends. J Food Eng 172, 25-30.

Vazquez-Ovando, A., Rosado-Rubio, G., Chel-Guerrero, L., Betancur-Ancona, D., 2009. Physicochemical properties of a fibrous fraction from Chia (*Salvia hispanica* L.). Food Sci. & Technol. 42, 168–173.

Vuksan, V., Jenkins, A.L., Dias, A.G., Lee, A.S., Jovanovski, E., Rogovik, A.L., Hanna, A., 2010. Reduction in postprandial glucose excursion and prolongation of satiety: possible explanation of the long-term effects of whole grain Salba (*Salvia Hispanica* L.). Eur. J. Clin. Nutr. 64, 436-438.

Wu, G., Johnson, S.K., Bornman, J.F., Bennett, S.J., Fang, Z., 2017. Changes in whole grain polyphenols and antioxidant activity of six sorghum genotypes under different irrigation treatments. Food Chem. 214, 199–207.

Zhang, J., Kirkham, M.B., 1996. Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytol 132, 361-373.

7. General conclusion

Metabolomic fingerprinting by spectroscopic and spectrometric techniques is a modern approach to study the whole metabolome of a biological sample. The purpose of this dissertation has been to demonstrate the usefulness of this approach coupled with chemometric analysis for sample classification and metabolite detection. Metabolomic analysis was carried out on two plants of great interest in food industry: the artichoke and sage.

In particular, for the first time, NMR spectroscopy and multivariate data analysis were used to define metabolite composition of different *Cynara* varieties. The obtained data confirmed the genetic distance between the edible globe artichoke and cardoon. Findings showed that an untargeted metabolomic approach may be an effective tool for chemotaxonomy classification when limited information are available. Moreover, in this research the level of nutraceuticals was found to be highest in Bianco di Pertosa zia E and Natalina landraces, which can be proposed as raw materials for the development of new functional products.

In the second part of this dissertation, a comparative analysis between commercial short-day flowering chia (*S. hispanica*) seeds and mutant genotypes was achieved. Results demonstrated how the metabolomic study can be applied on different biological samples to analyse the effect on the metabolite composition of external chemical or physical treatments, such as mutation, fertilization and irrigation. This approach also proved that chia seeds of mutant genotypes can be cultivated in other temperate areas at high latitude without loss in nutraceuticals. Metabolomics could be used as monitoring technique to control the agronomic management and its non-invasive features make it an ideal tool for pharmaceutical, agricultural and food industries.